A Study of Drug Resistance Mechanism in Human Carcinoma Cells after Hypoxia Exposure

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Abstract of the thesis entitled:

A Study of Drug Resistance Mechanism in Human Carcinoma Cells after Hypoxia Exposure

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Hypoxia is a common phenomenon observed in solid tumors and is mainly due to the improper development of vasculature. It was reported that hypoxia conferred resistance to cancer therapy, especially for chemotherapy and radiotherapy. However, there is not much work focused on the drug resistance in hypoxia/reoxygenation treated carcinoma cells. In the present study, the drug resistance mechanism in human carcinoma cells, HepG2 and A431, after hypoxia/reoxygenation treatment (G10HR, G20HR and A10HR and A20HR) were investigated.

The sensitivity in HepG2 and A431 cells to 13 drugs was examined and it was observed that hypoxia/reoxygenation treated cells were generally resistant to the selected drugs. Among them, the resistance mechanisms for cisplatin and doxorubicin were further studied in HepG2 and A431 cells respectively. Accordingly, the overexpression of P-glycoprotein in A10HR and A20HR cells was found to be related to the resistance to doxorubicin treatment. In addition, the expression of P-glycoprotein was suspected to be regulated by DNA methylation in the promoter region of mdr1 gene.

For the resistance mechanism to cisplatin in G10HR and G20HR cells, mismatch

repair gene PMS2 seemed to play an important role. The down-regulation of PMS2 was correlated with the cisplatin resistance. Also, our results showed that the expression of PMS2 may be regulated by both DNA methylation and histone deacetylation. Besides, reduced expressions of pro-apoptotic proteins and enhanced expressions of anti-apoptotic proteins were observed in G10HR and G20HR cells and this phenomenon also seemed to contribute for the reduction of cisplatin-induced apoptosis. Furthermore, PMS2 was found to act as a direct signaling molecule in cisplatin-induced apoptosis in HepG2 cells by RNA interference.

摘要

缺氧狀態是一個常見於堅實腫瘤的現象，而主要原因是脈管系統的異常發展。據報導，缺氧狀態會減少癌症療法的效果，特別是化療和放射療法。然而，關於缺氧狀態如何減少癌症療法的效果的研究並不多。在是次研究中，我們會就缺氧狀態如何令人類肝癌細胞 HepG2 和人類磷狀細胞癌 A431(G10HR, G20HR 和 A10HR, A20HR)對藥物產生抗藥的機制進行研究。

首先，我們挑選了十三種藥物對 HepG2 和 A431 癌細胞進行敏感度測試，結果發現，經過缺氧狀態處理的癌細胞普遍對藥物產生抗藥性。在當中，我們挑選了順鉑(cisplatin)和阿霉素(doxorubicin)分別在 HepG2 和 A431 癌細胞如何產生抗藥性的機制進行深入研究。 在阿霉素的研究中，我們發現 P-醣蛋白在 A10HR 和 A20HR 癌細胞的過度表達可能是對阿霉素產生抗藥性的主因。此外，經過脫氧核糖核酸甲基化作用(DNA methylation)的多藥耐藥基因(mdr1)的啟動子被懷疑是負責控制 P-醣蛋白的表達程度。

至於順鉑在 G10HR 和 G20HR 的抗藥性機制中，PMS2 似乎扮演了一個重要的角色。研究發現，減少 PMS2 的表達與順鉑的抗藥性有關。我們的結果亦發現 PMS2 的表達受著脫氧核糖核酸甲基化作用以及組織蛋白去乙醯基化作用(histone deacetylation)所控制。另外，在 G10HR 和 G20HR 癌細胞裡，促進凋亡蛋白的減少和抗凋亡蛋白的增加似乎是減輕了由順鉑引致的細胞凋亡的主因。最後，透過核糖核酸干擾方法(RNA interference)，我們展現了 PMS2 作為一個指導順鉑引致的細胞凋亡的訊號分子。

Abbreviations

5-Aza-dC	5'-aza-2'-deoxycytidine
ABC	ATP-binding cassette
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Apaf-1	Apoptotic Protease Activating Factor-1
APCs	Antigen-presenting cells
ATP	Adenosine triphosphate
BCP	1-bromo-3-chloropropane
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
DEPC	Diethyl cyanophosphonate
DHFR	Dihydrofolate reductase
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
DR	Death receptor
hEGFR-2	Human epidermal growth factor receptor-2
ERCC1	Excision repair cross-complementation group 1
EtBr	Ethidium bromide
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum

FGF-2	Fibroblast growth factor-2
GSC	Glucosylceramide synthase
GST	Glutathione transferases
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylases
HIF-1	Hypoxia-inducible factor-1
IAPs	Inhibitor of apoptosis proteins
IFNGR	Interferon gamma-receptor
IFNα	Interferon alpha
IFNγ	Interferon gamma
LED-Myocet®	Liposome-encapsulated doxorubicin-citrate complex
LRP	Lung resistance-related protein
MDR	Multidrug resistance
MGMT	O6-methylguanine-DNA methyltransferase
mmHg	millimeter Mercury
MMR	Mismatch repair
MRP	Multidrug resistance-associated protein
MSP	Methylation-specific PCR
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
NER	Nucleotide excision repair
ONOO(-)	Peroxynitrite
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C

PLD-Caelyx®	Pegylated liposomal doxorubicin
PMSF	Phenylmethanesulfonyl fluoride
PMS2	
RFC	Reduced folate carrier
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate
SMAC	Second mitochondrial-derived activator of caspase
STAT	Signal transducer and transactivator
Th1	CD4+ T helper cell type 1
TNF-R	Tumor-necrosis factor receptor
TNFα	Tumor necrosis factor-alpha
TOPOII	Topoisomerase II
TSA	Trichostatin A
WHO	World Health Organization

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Chapter 1

General Introduction

1.1 Introduction

Although many kinds of treatment approaches have been developed and administered in the therapy of cancer, the therapy is still not promising as none can have a 100% cure of cancer. Instead, development of resistance to the therapy often exists clinically, particularly for the advanced cancer. In fact, cellular heterogeneity, redundancy of molecular pathways and continuous changing in microenvironment may contribute to the survival, motility and metastasis of solid tumors. Moreover, the proliferation and progression of the tumor are likely dependent on more than one abnormally activated signaling pathway. Therefore, treatments interfering only a single target seem becoming less and less effective. In contrast, combined therapy is thought to be an attractive therapeutic strategy.

1.1.1 Treatment resistance in cancer

Treatment resistance in cancer can be intrinsic or acquired after therapy. Intrinsic resistance may be related to the epigenetic regulation of the cell physiology. For the acquired resistance, it may be contributed by the expressions of energy-dependent transporters, development of defect in drug-induced apoptosis, abnormal detoxification of drugs, enhanced DNA repair and cell cycle arrest. For instance, multidrug resistance-associated protein 4 (MRP4) ejected topotecan and conferred the resistance [Tian *et.al.*, 2006]; Polymorphism in the codon 72 of p53 was associated with shorter time of progression and overall survival in patients with metastatic breast cancer [Vannini *et.al.*, 2008]; Multidrug resistance has been linked to sphingolipid metabolism by glucosylceramide synthase (GCS) in breast cancer

[Ruckhaberle *et.al.*, 2008]; Enhanced nucleotide excision repair (NER) capacity led to cisplatin resistance [Wu *et.al.*, 2003]; glucocorticoids induced therapeutic resistance in malignant solid tumors by decreasing their proliferation rates [Mattern *et.al.*, 2007].

1.1.1.1 Surgery

Surgery is the most traditional clinical method to cure cancer by physically removal of the cancerous tissue. Although surgery alone usually cannot remove cancerous tissue completely and recurrence usually can happen, there are several benefits of surgery. First of all, it can eliminate the poorly vascularised tumor that is usually drug-resistant and malignant. In addition, it improves the perfusion in the small residual tumor mass that favors the function of chemotherapy. Furthermore, the host immunocompetence is enhanced. To achieve the optimal level of surgery treatment, there are different definitions for different cancers. For ovarian cancer, it refers to the complete debulking to no visible tumor residuals [du Bois and Harter, 2006]. Since surgery alone is not an effective method of treating cancer, it usually combines with other types of treatment such as chemotherapy or radiotherapy to achieve a better outcome. In a case study, a combination of surgery and adjuvant chemotherapy results in a complete and long-lasting remission of an aggressive recurrent melanotic neuroectodermal tumor of infancy in a 4-month-old male infant [Neven *et.al.*, 2008].

1.1.1.2 Chemotherapy

Chemotherapy refers to the use of drugs to kill cancerous cells. Mainly, it works by stopping cell proliferation or initiating cell death by damaging DNA directly or indirectly. In most cases, drugs were applied clinically without knowing their exact action mechanisms. Chemotherapeutic drugs can be categorized according to their function and origin. They include alkylating agents, which act by adding alkyl groups, antimetabolites, which interfere cell division, plant alkaloids, which disrupt microtubule function, topoisomerase inhibitors, which affect the transcription and replication of DNA. The major obstacle of chemotherapy is resistance and tremendous reports revealed the appearance of chemotherapeutic resistance in clinical cases. For example, clinical resistance to platinum-based chemotherapy in small cell lung cancer was related to the expression of MRP2 [Ushijima *et.al.*, 2007], copper-transportin P-type ATPase contributed resistance to camptothecin in clinical colon cancer [Owatari *et.al.*, 2007], enhanced nuclear metallothionein expression led to poor clinical outcome of cisplatin treatment in ovarian cancer [Surowiak *et.al.*, 2007]. Undoubtedly, the clinical resistance was unlimited to particular cancer and contributed by multiple reasons.

1.1.1.3 Radiotherapy

Apart from surgery and chemotherapy, radiotherapy is also one of the most common types of treatment for cancer. It makes use of the ionizing radiation to kill and control the growth of cancerous tissues. It can either be used for curative or adjuvant cancer treatment. The action mechanism of radiotherapy is simple and clear.

It works by damaging the DNA of cancer cells by photon, electron proton, neutron or ion beam. The effectiveness of radiotherapy depends on the availability of free oxygen in the cells. Hence, this therapy is usually failure in treating hypoxic solid tumors. Nowadays, radiation therapy has become a safer and more successful therapy due to the rapid development in imaging technique. More accurate treatment can be achieved by calculating the dose distributions, controlling the accelerator and multileaf collimator and checking patient set-up [Svensson *et.al.*, 2003]. Nonetheless, development of resistance to radiotherapy was frequently observed. Generally, the resistance mechanism was related to the alteration in cell cycle and apoptotic pathways. Regarding the cell cycle, cells at G2 or M phase are more sensitive to radiation. It was found that overexpression of cyclin B1 was associated with resistance to radiotherapy [Hassan *et.al.*, 2002]. For the apoptotic pathways, Imoto I suggested that the overexpression of cIAP1 was correlated with radiotherapeutic resistance in cervical cancer [Imoto *et.al.*, 2002]. Recently, the glucose metabolism was found to affect the effectiveness of radiotherapy in squamous cell carcinoma of the oral cavity [Kunkel *et.al.*, 2007].

1.1.1.4 Hormonal therapy

Hormonal therapy was restricted to limited types of cancers with hormonally responsive tissues such as breast cancer and prostate cancer. The principle of this therapy is the manipulation of the endocrine system by the administration of hormones or interference with the function of hormone. Rarely, hormonal therapy is used solely to cure cancer. Instead, it is used as a supplement for other treatment and aimed at preventing or delaying the recurrence of cancer. Clinically, hormone can be

injected into the patient's body orally, intravenously or intramuscularly. The resistance to hormonal therapy was reported clinically. Survivin, a new member of IAP family, conferred resistance to antiandrogen therapy through IGF-1 signaling [Zhang *et.al.*, 2005]. The overexpression of human epidermal growth factor receptor-2 (HER-2) weakened the antiproliferative effect of endocrine therapy for breast cancer [Dowsett, 2001].

1.1.2 Hypoxia/reoxygenation and its correlation with treatment resistance

When a tumor proliferates much faster than the vasculature, it will not receive enough oxygen due to diffusion limit of oxygen within tissues. This phenomenon is called hypoxia. Normally, it refers to a condition in which the oxygen tension is less than 10mmHg (1.3%) and usually found in solid tumors. If the oxygen level drops even lower, the tumor cells will become necrotic and die (Fig. 1.1.1). Occasionally, the hypoxic region of the solid tumor may become oxygenated again due to the formation of new blood vessels. The physiology of hypoxic cells is somehow different from the hypoxia/reoxygenated cells. Adenosine triphosphate (ATP) is lost in ischemic tissues and results in failure of anaerobic glycolysis and the cells die through necrosis. On the other hand, reperfusion restores the ATP pool inside the cells that lead to additional losses of cells through apoptosis [Saikumar *et.al.*, 1998]. One group of scientists revealed that the reoxygenation following hypoxia protected myocytes against apoptosis and increased the survival rate of myocytes and this was contributed by the reduced peroxynitrite (ONOO(-)) formation [Wang *et.al.*, 2006]. Since the effect of reoxygenation following hypoxia remained unclear, further work was therefore needed.

The hypoxic tumor usually inherits resistance towards various kinds of therapies. It is less sensitive to radiotherapy due to the decrease in oxygen-free radicals which are the main component to produce DNA damage to cells. Brizel DM has pointed out the tumor hypoxia adversely affects short term clinical radiation response of head and neck cancer lymph node metastases [Brizel *et.al.*, 1997]. Also, the tumor is resistant to chemotherapy because of limited delivery of drugs by circulation.

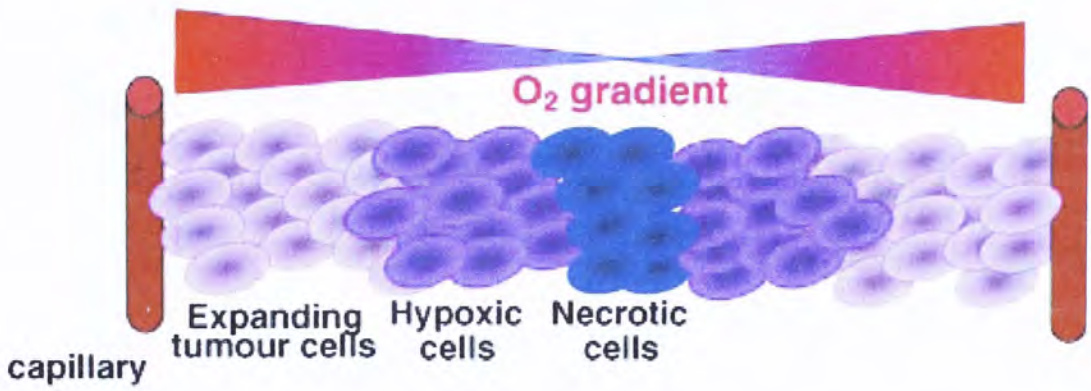


Fig. 1.1.1 A schematic diagram representing how the limited oxygen diffusion creates the hypoxic and necrotic regions in solid tumor. [Brahimi-Horn *et.al.*, 2007]

1.1.3 Aim of the study

As scientists become more and more concerned about the relationship between the condition of microenvironment and the treatment resistance in solid tumors, the objective of the present study is to figure out the resistance mechanisms to chemotherapy in hypoxia/reoxygenated cancer cells. Throughout the study, two human cancer cell lines were used: hepatocellular carcinoma cells (HepG2) and squamous carcinoma cells (A431). First of all, the sensitivity of

hypoxia/reoxygenated cells towards various kinds of drugs were examined (Chapter 2) so as to find out if there were any differences in the susceptibility to the drugs between hypoxia/reoxygenated cells and their parental cells. Interestingly, hypoxia/reoxygenated cancer cells were found to be resistant to several drugs. Among all, the resistance mechanisms of two selected drugs, doxorubicin and cisplatin, were further investigated (Chapter 3 and 4) respectively. Besides their frequent use clinically in treating a broad range of cancers, numerous reports of treatment resistance also urged us to examine the mechanism of treatment resistance. It was suggested that hypoxia/reoxygenated cancer cells underwent a different apoptotic pathway. Therefore, it is worth knowing whether this difference could lead to resistance towards particular drugs. Interesting results were obtained and elucidated (Chapter 5). All in all, it was hoped that the experimental findings could help us to improve the effectiveness of cancer treatment by modifying the present therapy or designing novel target-specific drugs.

Chapter 2

The Drug Sensitivity in HepG2 Cells and A431 Cells

2.1 Introduction

According to the World Health Organization (WHO), 7.6 million people died from cancer which accounted for 13% of the total number of deaths worldwide in 2005. There is an urge to develop suitable treatments for this leading and devastating disease so as to save more lives. Among all types of cancer, lung, stomach, liver, colorectal and oesophagus cancers are the top five common cancers for male while breast, lung, stomach, colorectal and cervical cancers are the most frequent cancer types for female.

2.1.1 Treatment of cancer

In order to deal with cancers, various kinds of treatments are adopted nowadays including surgery, chemotherapy, radiotherapy and immunotherapy. If the tumor is in an early stage without metastases, surgery is the preferred option for treatment. For instance, removal of tumors of the pancreatic head and the periampullary region using pancreaticoduodenectomy [Michalski *et.al.*, 2007]. However, surgery alone often cannot help curing cancer completely and so other kinds of therapies are chosen.

Chemotherapy refers to the use of drugs to cure cancer. It works by killing cells through triggering apoptosis, or, stopping or retarding growth of cancer cells through interfering cell division. Except using chemotherapy alone, it is usually administered along with other therapy. Sometimes, chemotherapy reduces the size of tumor before subjecting to surgery or radiation therapy and this is called neo-adjuvant chemotherapy. Conversely, if drugs were taken after surgery or radiation therapy, this is called adjuvant chemotherapy. Although chemotherapy is so effective

in treating neoplastic diseases, side effects often exist such as nausea, vomiting and hair loss. In a worse case, drug resistance may even appear.

2.1.2 Drug resistance

The anticancer drug resistance can be divided into two classes: host factors and genetic or epigenetic alterations in the cancer cells. The phenomenon involving simultaneous resistance to unrelated drugs is called Multidrug resistance (MDR). The mechanisms of MDR are not yet well defined. Diminishing of intracellular drug accumulation by ATP-binding cassette (ABC) transporters such as P-glycoprotein and multidrug resistance-associated protein (MRP), drug detoxification by glutathione-S-transferase, altered targets such as topoisomerase II and modification in apoptosis such as the Bcl-2 pathway have been implicated in the development of the phenotype of MDR [Ling, 1997]. Host factors refer to the poor absorption or delivery, and, enhanced metabolism or excretion of a chemotherapeutic agent. In addition, the environmental conditions around the tumors like the extracellular matrix or tumor geometry also contribute to drug resistance [Perez-Tomas, 2006]. Other than MDR, the microenvironment is also an important factor in inducing drug resistance. Acidosis, hypoxia and increased glycolysis are the typical host factors commonly found in the tumor microenvironment.

In this project, the relationship between hypoxia/reoxygenation and the sensitivity of chemotherapy were studied including the resistance mechanism of particular drugs in order to help designing novel drugs or new treatment for the chemotherapy-resistant cancers. Human hepatocellular carcinoma HepG2 cells and human squamous carcinoma A431 cells were used in the present study.

2.2 Materials and Methods

2.2.1 Cell culture

Human hepatocellular carcinoma HepG2 cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen , Carlsbad CA, USA) while human squamous carcinoma A431 cells were supplemented with 10% fetal bovine serum. The cells were incubated in humidified incubator with 10% CO_2 at 37°C.

For HepG2 cells, two hypoxia/reoxygenation sublines, G10HR and G20HR, were prepared. Firstly, the HepG2 cells were subjected to hypoxia for 96 hours followed by reoxygenation and regrow to 70% confluence. Then, the cells were passed twice under normoxia. Afterwards, the cells were subjected to the hypoxia/reoxygenation treatment again. The G10HR cells were derived from 10 cycles of hpoxia/reoxygenation treatment, while G20HR cells were derived from 20 cycles of hypoxia/reoxygenation treatment. Similarly, two hypoxia/reoxygenation sublines, namely A10HR and A20HR, were prepared. The A10HR cells were derived from 10 cycles of hpoxia/reoxygenation treatment, while A20HR cells were derived from 20 cycles of hypoxia/reoxygenation treatment. Stocks of all the cell lines were prepared and stored in liquid nitrogen tank. The cell lines were discarded and thawed from the stock when they reached passage 15. All the assays beyond were performed in air.

2.2.2 Drugs

Cisplatin, carboplatin, doxorubicin, etoposide, hydrogen peroxide, methotrexate, paclitaxel and vincristine were purchased from Sigma. Camptothecin, 10-hydroxy

camptothecin and TNF alpha were purchased from Merck. Interferon alpha (IFNα) and interferon gamma (IFNγ) were purchased from Millipore.

2.2.3 MTT assay

To test the sensitivity of cells towards different kinds of drugs, MTT cell survival assay was performed. Briefly, the cells were seeded in 96-well plates with a density of 1500 cells/well for HepG2 cells or a density of 500 cells/well for A431 cells for 2 days and then drugs with various concentrations were added to the cells and incubated for 5 days. After the drug treatment, the medium in the wells was aspirated and replaced with 50μl 1mg/ml 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) solution. The cells were incubated for an additional 3 hours so that the yellow MTT solution can be reduced to insoluble purple formazan by the mitochondrial dehydrogenase which was present in the viable cells. Finally, 150μl dimethyl sulfoxide (DMSO) was added to each well in order to dissolve the insoluble purple formazan and form a colored solution. The absorbance of each well was measured at 570nm by a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.3 Results

2.3.1 The drugs to which G10HR and G20HR cells were more resistant

From the results in Fig.2.3.1a and 2.3.1b, HepG2 cells responded to cisplatin, carboplatin, camptothecin, 10-hydroxy camptothecin and methotrexate in a dose-dependent manner. Furthermore, G10HR and G20HR cells were more resistant to all of them than the parental HepG2 cells (GP). The IC50 of cisplatin for G10HR and G20HR cells were about 2.88- and 2.63-fold of that for the GP cells, respectively. The IC50 of carboplatin for G20HR cells were about 1.66-fold of that for GP cells while that of G10HR cells was more than 2-fold. The IC50 of camptothecin for G10HR and G20HR cells were about 1.22- and 1.31-fold of that for GP cells, respectively. The IC50 of 10-hydroxy camptothecin for G10HR and G20HR cells were both more than 2-fold of that for GP cells. The IC50 of methotrexate for G10HR and G20HR cells were about 1.35 and 1.30 fold of that for GP cells respectively.

2.3.2 The drugs of which GP, G10HR and G20HR cells have similar response

From the results in Fig.2.3.2 and 2.3.3, all the three HepG2 cell lines responded more or less the same to etoposide, hydrogen peroxide, paclitaxel, TNF alpha and vincristine in a dose-dependent manner. It is interesting that TNF alpha seemed not to pose any lethal effect on all the three HepG2 cell lines under the selected range of concentration.

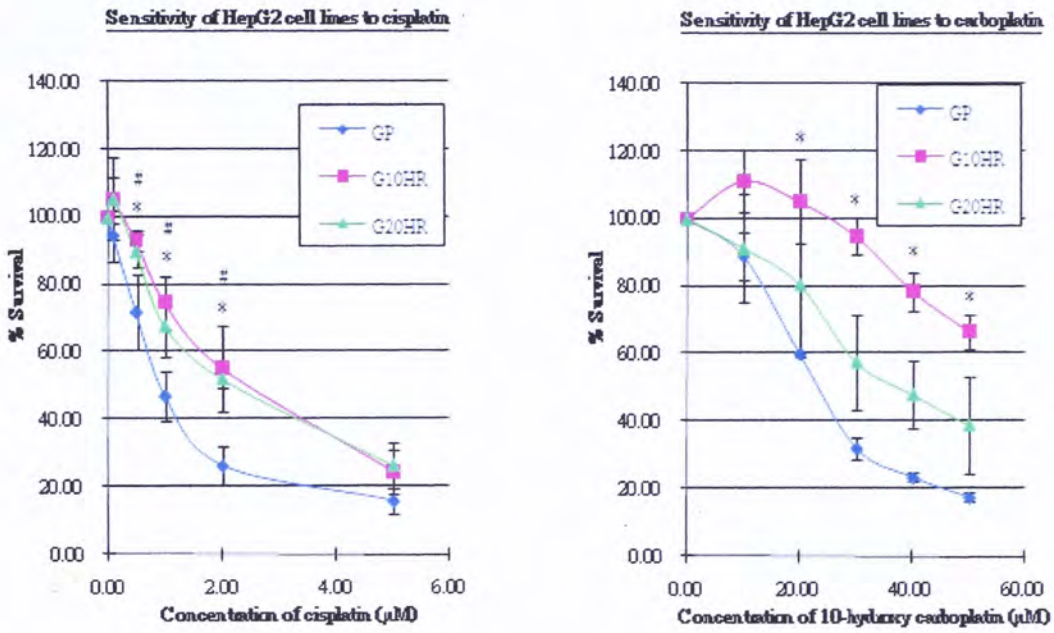


Fig. 2.3.1a. The sensitivity of HepG2 cells to cisplatin and carboplatin as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

*: $p < 0.05$ (between AP and A10HR); #: $p < 0.05$ (between AP and A20HR)

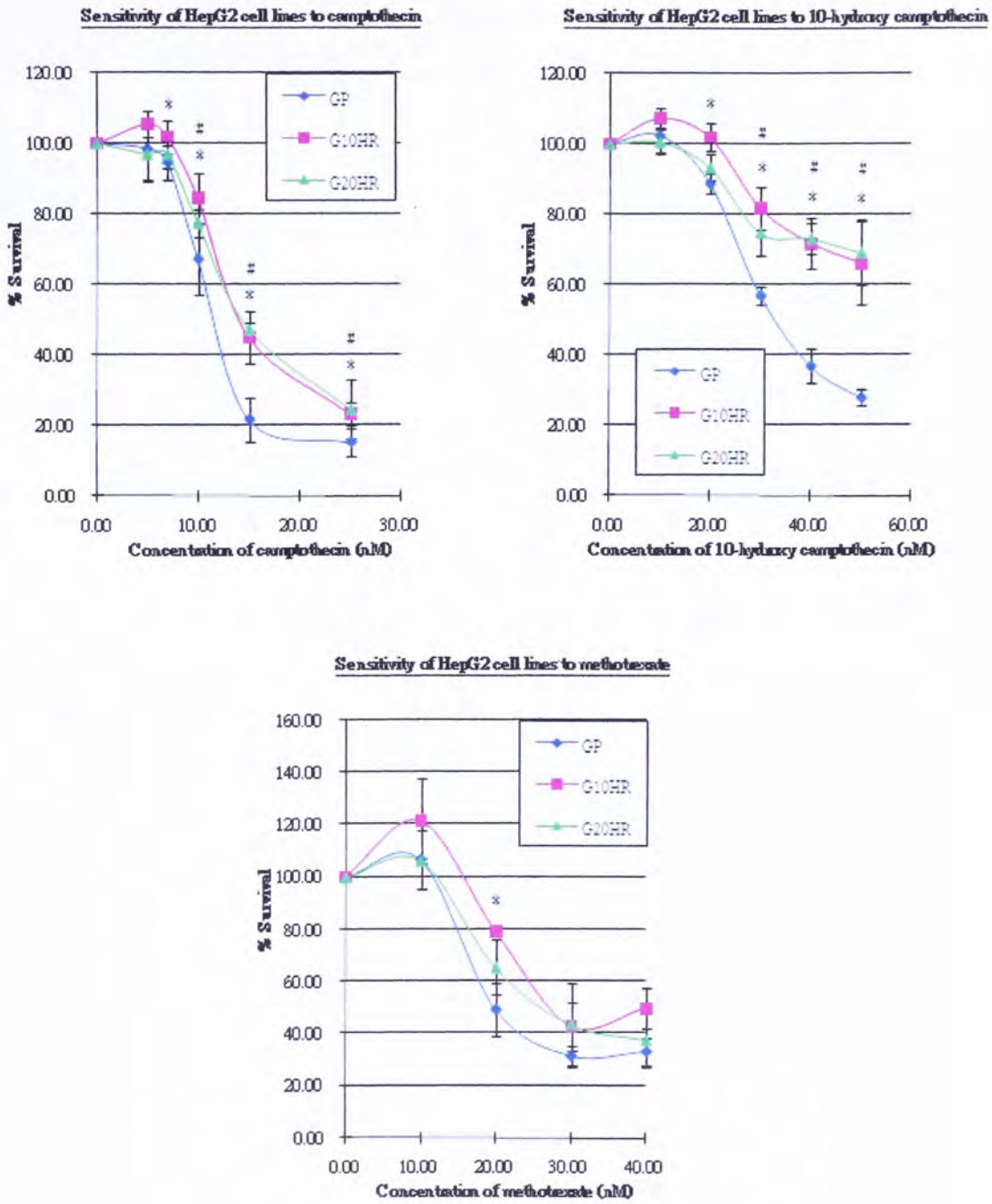


Fig. 2.3.1b. The sensitivity of HepG2 cells to camptothecin, 10-hydroxy camptothecin and methotrexate as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; -- ▲ -- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments. *: $p < 0.05$ (between AP and A10HR); #: $p < 0.05$ (between AP and A20HR)

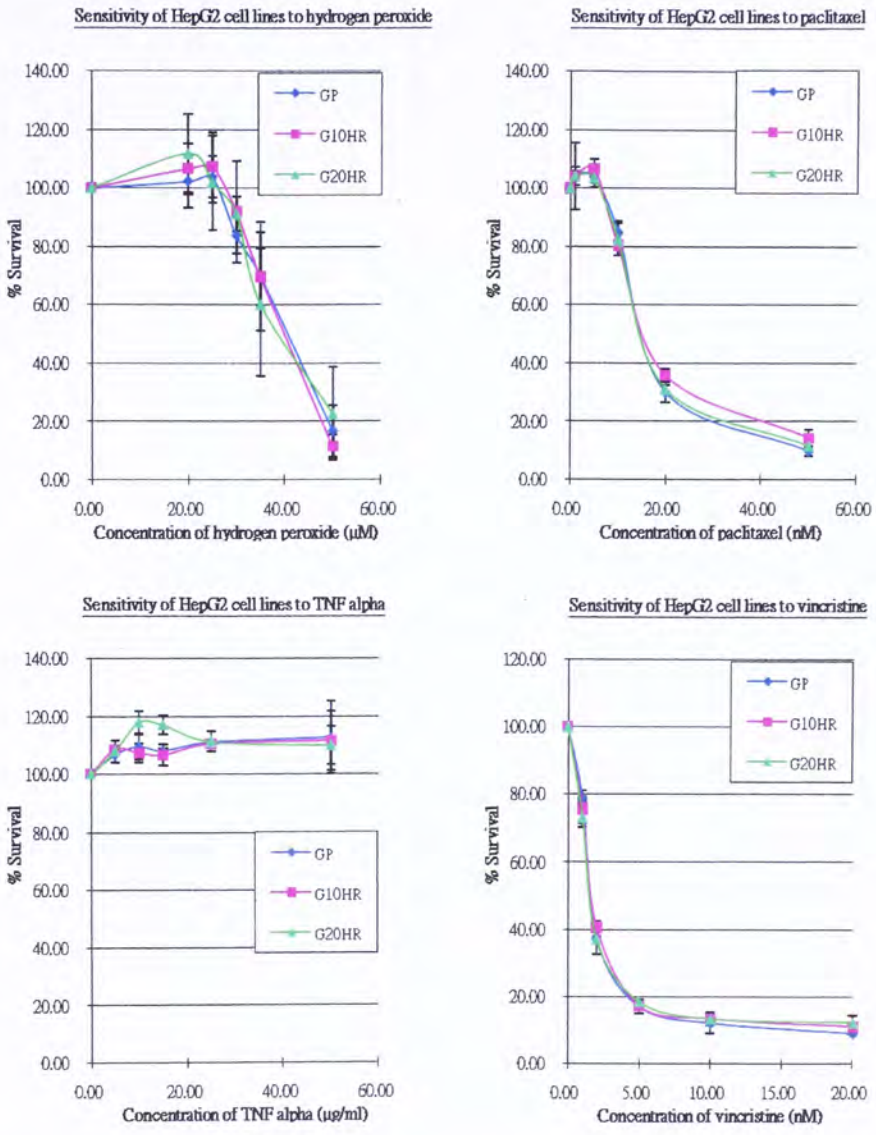


Fig. 2.3.2. The sensitivity of HepG2 cells to hydrogen peroxide, paclitaxel, TNF alpha and vincristine as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

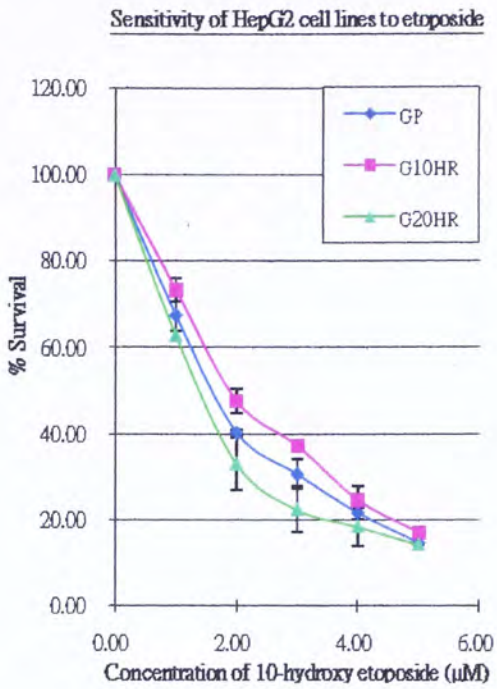


Fig. 2.3.3. The sensitivity of HepG2 cells to etoposide as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; -- ▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

2.3.3 The drugs to which A10HR and A20HR cells were more resistant

From the results in Fig 2.3.4a and 2.3.4b, A431 cells responded to carboplatin, doxorubicin, hydrogen peroxide and IFNα in a dose-dependent manner. Besides, A10HR and A20HR cells were more resistant to all of them than their parental A431 cells (AP). The IC50 of carboplatin for A10HR and A20HR cells were both more than 2.5-fold of that for AP cells. The IC50 of doxorubicin for A10HR and A20HR cells were about 1.08-fold and 1.21-fold of that for AP cells respectively. The IC50 of hydrogen peroxide for A10HR and A20HR cells were about 1.10- and 1.32-fold of that for AP cells respectively. The IC50 of IFNα for all the three A431 cell lines was undefined under the tested range of concentration.

2.3.4 The drugs to which A10HR and/or A20HR cells were more sensitive

From the results in Fig 2.3.5, A20HR cells were more sensitive to camptothecin and vincristine than that of AP cells while both A10HR and A20HR cells were more sensitive to methotrexate. The IC50 of camptothecin and vincristine for A20HR cells was about 0.63-fold and 0.55-fold of that for AP cells respectively. The IC50 of methotrexate for A10HR and A20HR cells were about 0.67-fold and 0.53-fold of that for AP cells respectively.

2.3.5 The drugs which AP, A10HR and A20HR cells have similar response

From the results in Fig 2.3.6a and 2.3.6b, all the three A431 cell lines responded more or less the same to 10-hydroxy camptothecin, cisplatin, etoposide, IFNγ, paclitaxel and TNF alpha in a dose-dependent manner. Similar to HepG2 cells, TNF alpha seemed also to have less killing on any of the three A431 cell lines under the selected range of concentration.

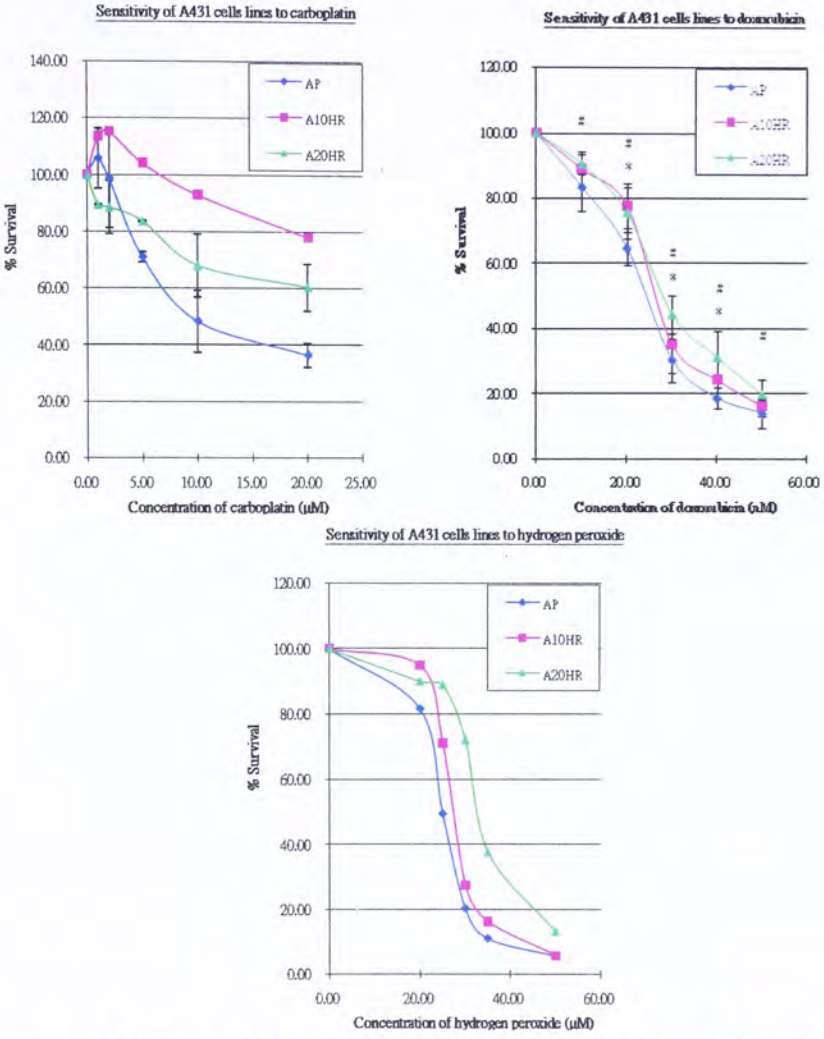


Fig. 2.3.4a. The sensitivity of A431 cells to carboplatin, doxorubicin and hydrogen peroxide as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control.

*: $p < 0.05$ (between AP and A10HR); #: $p < 0.05$ (between AP and A20HR)

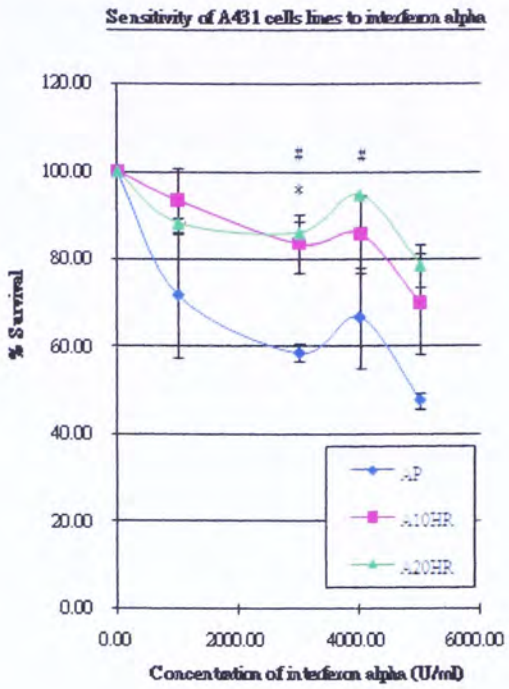


Fig. 2.3.4b. The sensitivity of A431 cells to interferon alpha as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control.

*: $p < 0.05$ (between AP and A10HR); #: $p < 0.05$ (between AP and A20HR)

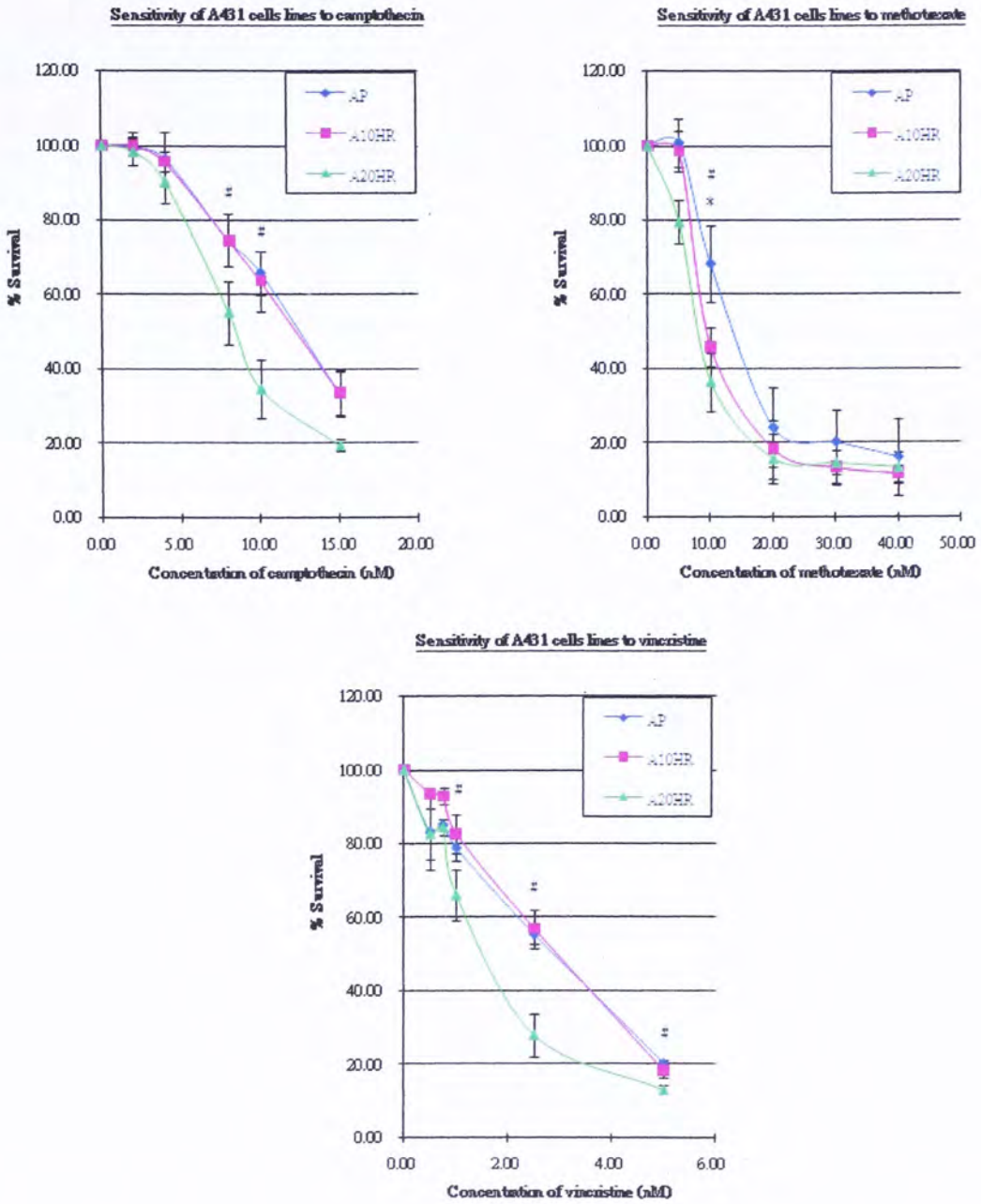


Fig. 2.3.5. The sensitivity of A431 cells to camptothecin, methotrexate and vincristine as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments. *: $p < 0.05$ (between AP and A10HR); #: $p < 0.05$ (between AP and A20HR)

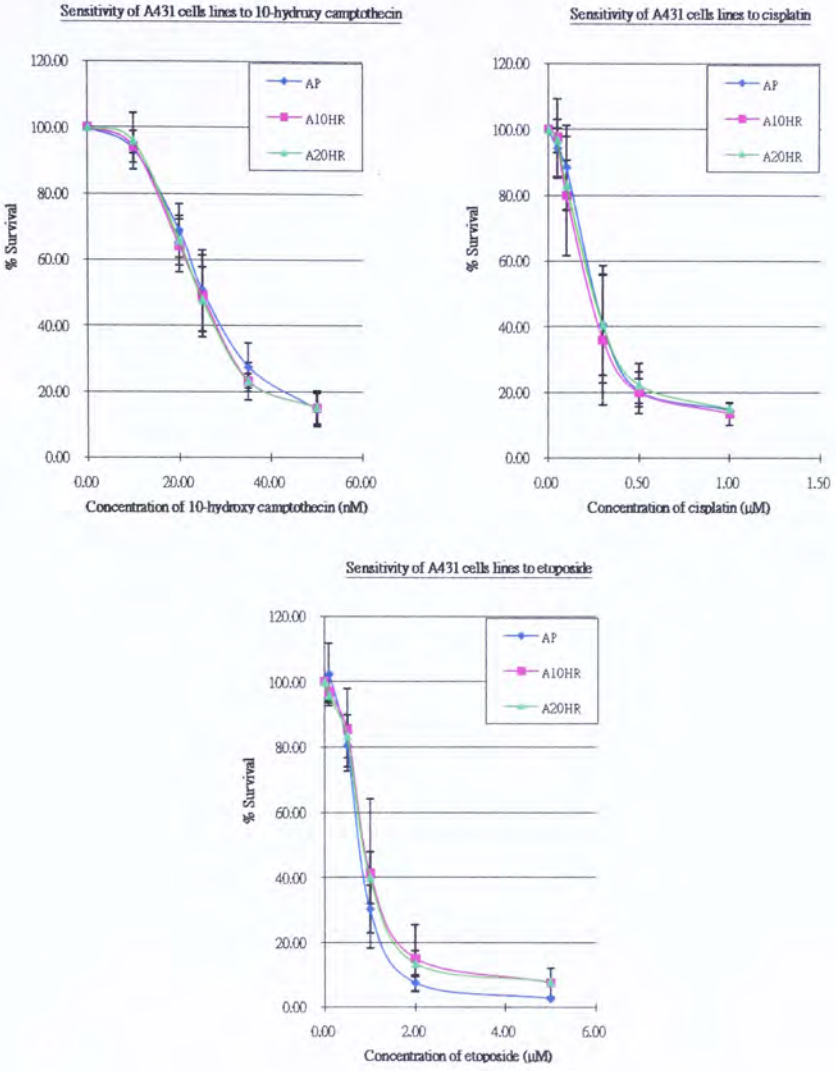


Fig. 2.3.6a. The sensitivity of A431 cells to 10-hydroxy camptothecin, cisplatin and etoposide as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

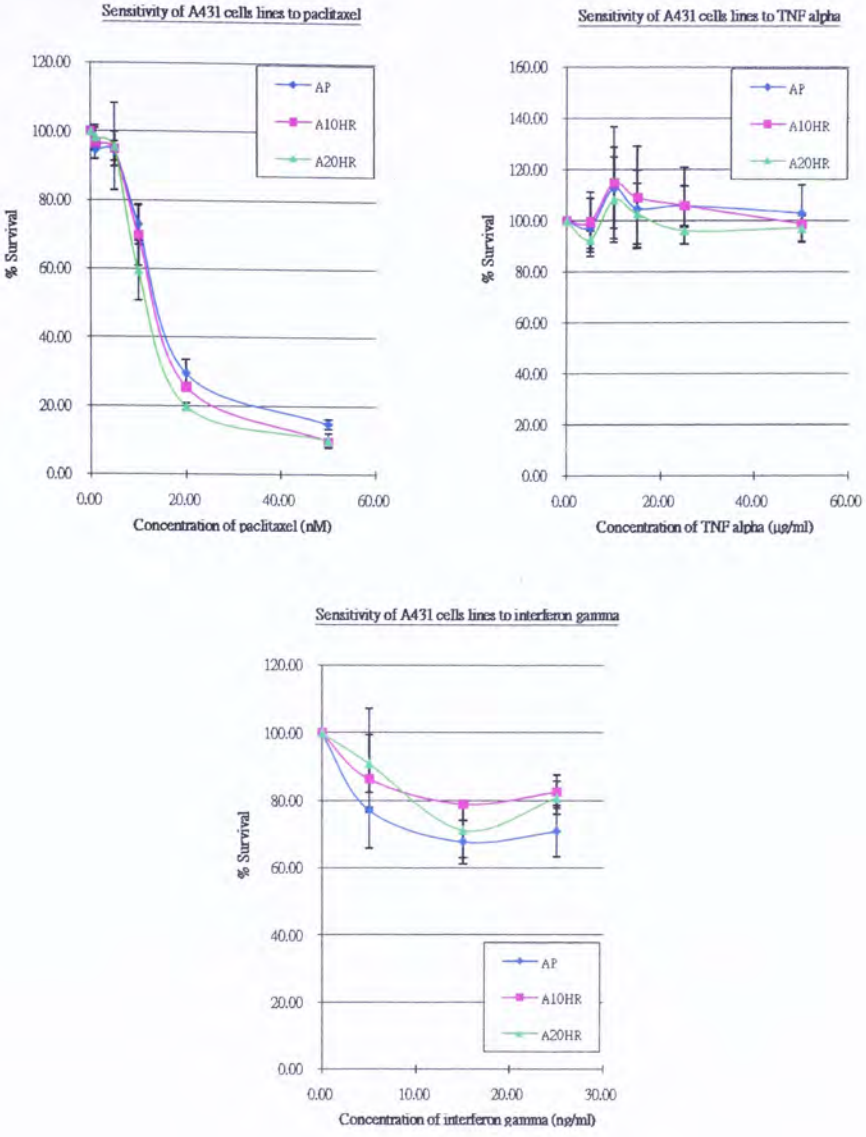


Fig. 2.3.6b. The sensitivity of A431 cells to interferon gamma, paclitaxel and TNF alpha as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

2.4 Discussion

Hypoxia/reoxygenation in solid tumors may change the physiology of carcinoma cells that make them even more adapt to the environment and survive. The altered gene and protein expressions probably contribute to the resistance towards different kinds of anticancer therapies. In order to have an insight on how to tackle the problem of chemotherapeutic resistance in solid tumors, the sensitivity to various kinds of drugs including those common in clinical use were studied in the selected cell lines. It consisted of platinum drugs (cisplatin and carboplatin), topoisomerase I inhibitors (camptothecin and 10-hydroxy camptothecin), topoisomerase II inhibitor (doxorubicin and etoposide), cytokines (interferon alpha, interferon gamma and tumor necrosis factor-alpha), antifolate drug (methotrexate), mitotic inhibitors (paclitaxel and vincristine) and oxidizing agent (hydrogen peroxide).

The results obtained from the experiments revealed that both A431 cells and HepG2 cells had similar sensitivity towards tumor necrosis factor-alpha (TNFα) and paclitaxel no matter the cells were subjected to hypoxia/reoxygenation treatment or not. This was also true for the sensitivity of A431 cells towards interferon gamma. However, either or both of the HepG2 cells and A431 cells showed resistance towards other drugs to a certain extent. For doxorubicin resistance, it will be discussed in Chapter 3. For platinum drug-resistance, it will be discussed to Chapters 4 and 5. For the remaining drugs, their structures, usages and resistance mechanisms will be discussed in the following. Table 2.4.1 showed the IC50 of various drugs tested in HepG2 cells and A431 cells while Table 2.4.2 summarized the sensitivity of drugs in HepG2 and A431 cells.

Table 2.4.1. The IC50 of 13 drugs tested in HepG2 cells and A431 cells.

	GP	G10HR	G20HR	AP	A10HR	A20HR
Cisplatin	0.8μM	2.3μM	2.1μM	0.23μM	0.23μM	0.25μM
Camptothecin	10.8nM	13.2nM	14.2nM	12.8nM	12.0nM	8.0nM
10-hydroxy camptothecin	33.0nM	>50.0nM	>50.0nM	26.4nM	27.0nM	27.4nM
Carboplatin	23.5μM	>50.0μM	39.0μM	9.0μM	>20.0μM	>20.0μM
Doxorubicin	ND	ND	ND	24.0nM	26.0nM	29.0nM
Etoposide	1.6μM	2.0μM	1.4μM	0.78μM	0.88μM	0.92μM
H_2O_2	40.5μM	40.5μM	39.0μM	25.0μM	27.5μM	33.0μM
Interferon alpha	ND	ND	ND	>5000 U/ml	>5000 U/ml	>5000 U/ml
Interferon gamma	ND	ND	ND	>50.0 ng/ml	>50.0 ng/ml	>50.0 ng/ml
Methotrexate	20.0nM	27.0nM	26.0nM	15.0nM	10.0nM	8.0nM
Paclitaxel	15.0nM	15.0nM	15.0nM	13.8nM	13.2nM	11.8nM
TNF alpha	>50.0 μg/ml	>50.0 μg/ml	>50.0 μg/ml	>50.0 μg/ml	>50.0 μg/ml	>50.0 μg/ml
Vincristine	1.6nM	1.6nM	1.6nM	2.9nM	2.9nM	1.6nM

Table 2.4.2. A summary of the sensitivity of 13 drugs in HepG2 and A431 cells.

	GP	G10HR	G20HR	AP	A10HR	A20HR
Cisplatin						
Camptothecin						
10-hydroxy camptothecin						
Carboplatin						
Doxorubicin	Nil	Nil	Nil			
Etoposide						
H_2O_2						
Interferon alpha	Nil	Nil	Nil			
Interferon gamma	Nil	Nil	Nil			
Methotrexate						
Paclitaxel						
TNF alpha						
Vincristine						

Less resistant

More resistant

Response similarly

(The table was constructed according to the value of IC50 and the graph plotted.)

2.4.1 Camptothecin and 10-hydroxy camptothecin

Camptothecin is a pentacyclic alkaloid extracted from *Camptotheca acuminate*. It is a topoisomerase I inhibitor and kills carcinoma cells by stabilization of topoisomerase I cleavage complex and the formation of permanent DNA strand breaks. As shown in Fig. 2.4.1, camptothecin consists of a closed α–hydroxyl-δ-lactone ring which is the key component for cytotoxicity. As it is poorly soluble in aqueous media and inactivated easily by lactone ring hydrolysis, many different derivatives of camptothecin with improved solubility and potency have been developed like topotecan, irinotecan and SN-38 (Fig. 2.4.2). The action mechanism of camptothecin is primarily by the formation of a covalent topoisomerase-I-camptothecin-DNA ternary complex known as the cleavage complex. This reversible complex cleavage is toxic to the cell only when it is collided with the replication fork. Consequently, a double-strand break formed that leads to an irreversible arrest of DNA replication at the replication fork. The cell death mechanism is then triggered (Fig. 2.4.3) [Liu *et.al.*, 2005]. 10-hydroxy camptothecin is one of the novel analogs of camptothecin which has higher anticancer activity and more effective in multidrug-resistant cancers.

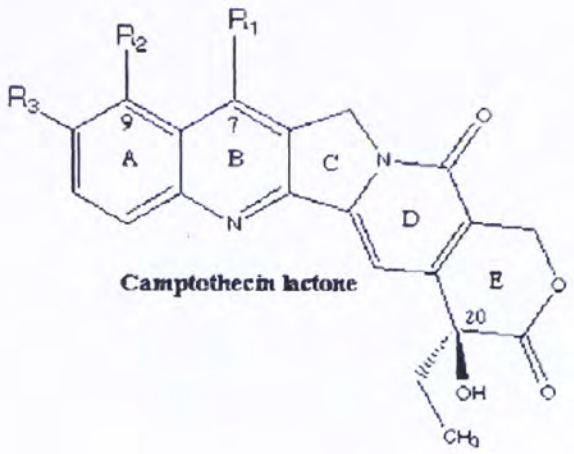


Fig. 2.4.1. The structure of camptothecin [Rasheed and Rubin, 2003].

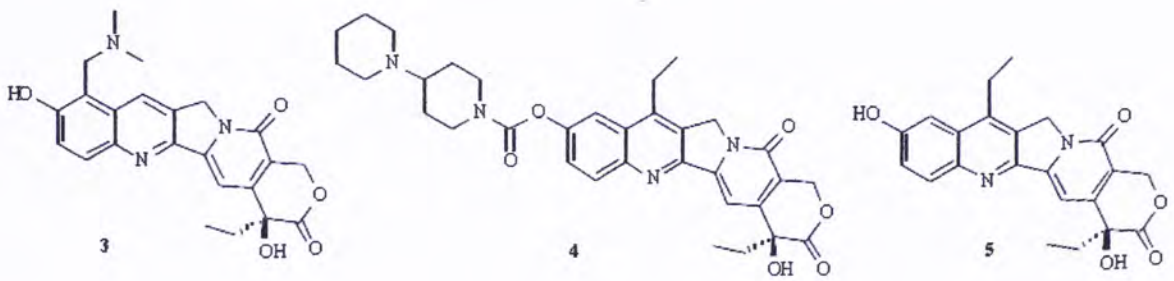


Fig. 2.4.2. The structures of topotecan, irinotecan and SN-38. [Li *et.al.*, 2006]

Although it is so effective in treating a broad spectrum of cancers, resistance has been reported with the following proposed reasons: (1) overexpression of efflux pump ABCG2 [Ishikawa *et.al.*, 2006]; (2) mutations in topoisomerase I [Chrencik *et.al.*, 2004]; (3) cytotoxicity modulation by DNA repair enzyme O(6-methylguanine DNA methyltransferase [Kuo *et.al.*, 2006]; (4) downregulation of topoisomerase I [Eng *et.al.*, 1990]; (5) alterations in the cellular response to the cleavage complex [Rasheed and Rubin, 2003].

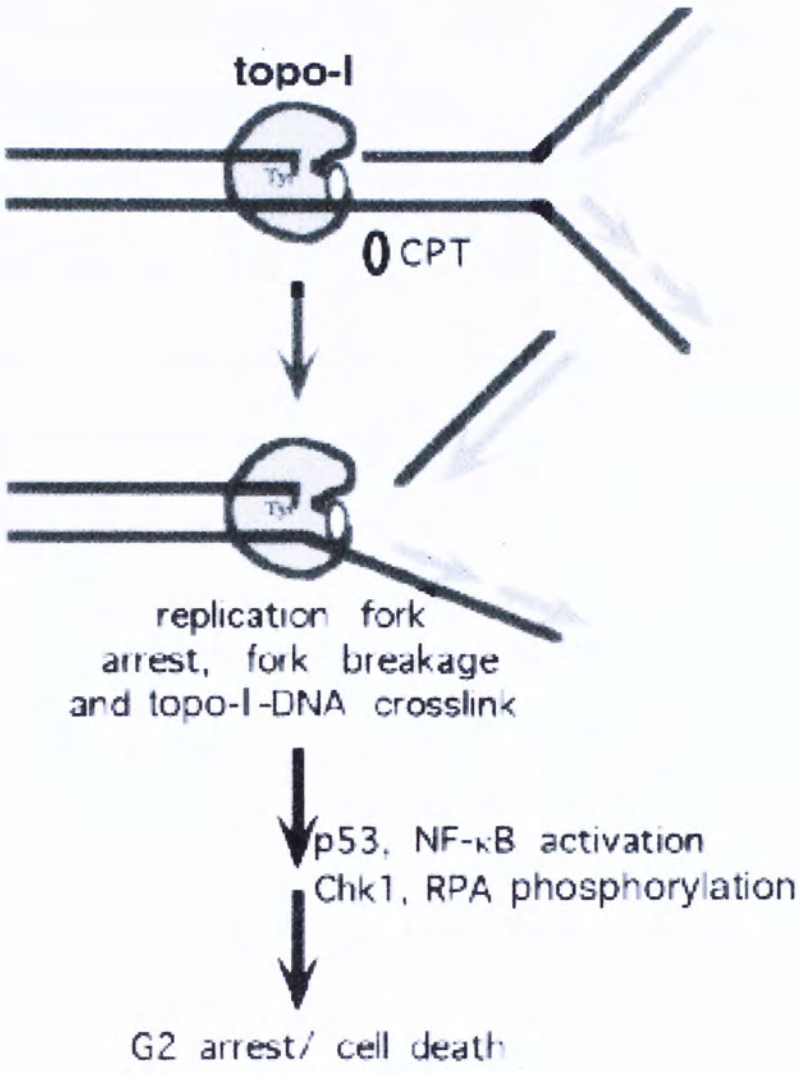


Fig. 2.4.3. Action mechanism of camptothecin [Liu *et.al.*, 2005].

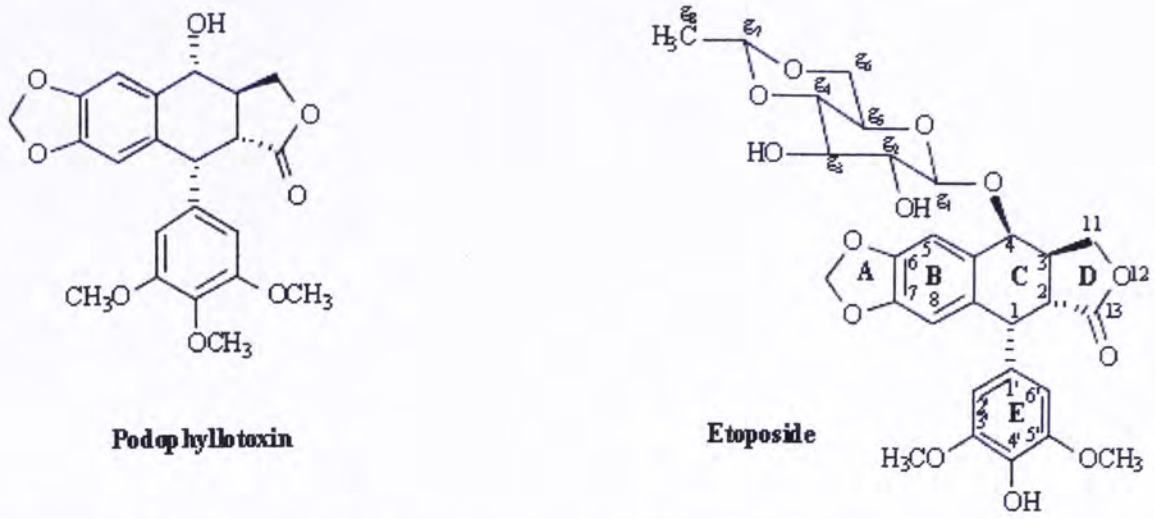


Fig. 2.4.4. Structure of podophyllotoxin and etoposide. Etoposide consists of a glycosidic group substituted at the C-4 position, a polycyclic core (A-D rings) and a pendant ring (E-ring) [Baldwin and Osheroff, 2005].

2.4.2 Etoposide

Etoposide is derived from a natural product called podophyllotoxin (Fig. 2.4.4). It has been used to treat a variety of cancers such as small cell lung cancer, germ-line malignancies and leukemias. Its main target remains as topoisomerase II and works by stabilizing a covalent enzyme-cleaved DNA complex. In details, etoposide approaches a topoisomerase-DNA ternary complex through interacting directly with the ATP-bound topoisomerase II monomer and forms single-stranded or double-stranded DNA breaks at sites that have a cytosine one base 5' to the cleavage bond. Attaching to the complex, etoposide inhibits the ligation activity of topoisomerase II and the cleavage complexes are accumulated. Consequently, recombination/repair pathways, mutagenesis and chromosomal translocations are triggered. If the damage cannot be recovered, apoptosis will then be induced (Fig. 2.4.5) [Baldwin and Osheroff, 2005, Montecucco and Biamonti, 2007].

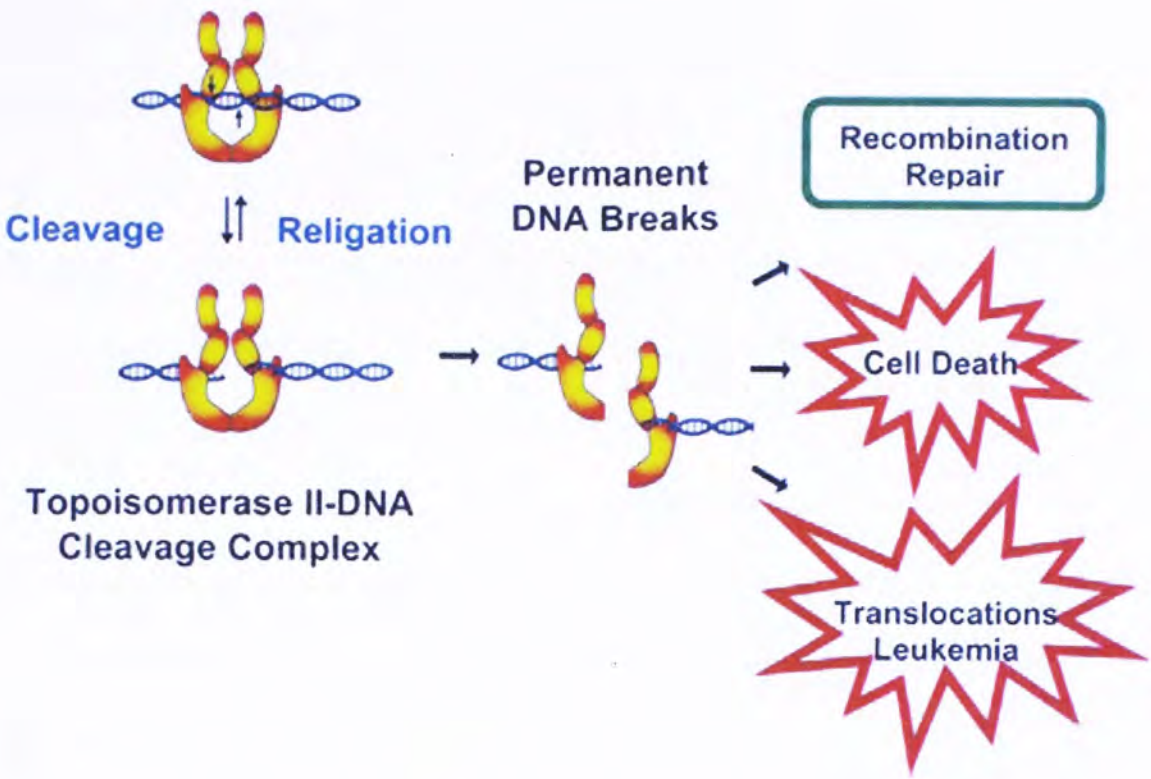


Fig. 2.4.5. A schematic diagram showing the effects of topoisomerase II-DNA cleavage complexes in cell [Baldwin and Osheroff, 2005].

The mechanism of etoposide resistance has been studied for a long time but it is still poorly understood. The mechanisms suggested include: (1) downregulation of topoisomerase II [Andoh *et.al.*, 1996]; (2) mutation of topoisomerase II [Chan *et.al.*, 1993]; (3) overexpression of multidrug resistance-associated protein (MRP) gene [Schneider *et.al.*, 1994]; (4) prevention of etoposide-induced apoptosis by fibroblast growth factor-2 (FGF-2) [Pardo *et.al.*, 2002]; (5) induction of hypoxia-inducible factor-1 (HIF-1) alpha [Hussein *et.al.*, 2006]; (6) loss of putative tumor suppressor gene EI24/PIG8 [Mork *et.al.*, 2007].

2.4.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a kind of reactive oxygen species (ROS) normally found in all aerobic organisms. It is mainly generated in mitochondria during oxidative phosphorylation and broken down by catalase and glutathione peroxidase. Hydrogen peroxide not only play a role in malignant transformation but can also play a role in triggering cell death. For carcinogenesis, hydrogen peroxide acts by inducing DNA damage, mutations and genetic instability. Nonetheless, hydrogen peroxide can also induce apoptosis in tumor cells. Moreover, experimental data have shown that drugs such as arsenic trioxide, which can increase the cellular levels of hydrogen peroxide, can effectively kill cancers cells. Although the issue is still controversial and direct administration of hydrogen peroxide was believed to be an inappropriate therapeutic strategy, the upregulation of cellular hydrogen peroxide by hydrogen peroxide-generating systems may be an alternative anticancer strategy [López-Lázaro, 2007].

2.4.4 Interferons (IFN)

Interferons are proteins secreted in response to stimuli and consist of at least five classes, being alpha, beta, gamma, tau and omega. This superfamily can be divided into two groups. Type 1 IFN consists of IFNα, IFNβ, IFNω, and IFNτ while type II only has IFNγ [Caraglia *et.al.*, 2005]. Traditionally, these proteins are used to protect the host against viral infection. However, they are becoming an important anticancer agent.

2.4.4.1 Interferon alpha (IFNα)

As mentioned before, IFNα is a type I interferon composed of 13 subtypes and is secreted nearly by all cell types. Nevertheless, all the subtypes share the same receptor system and function similarly. Apart from treating viral diseases like hepatitis C, IFNα was also used to treat hematological malignancies and solid tumors. Accordingly, the action mechanism of IFNα is believed to directly inhibit the tumor cell proliferation in several ways including downregulation of oncogene suppression and upregulation of tumor suppressor genes. Additionally, IFNα also enhances the immune response by induction of MHC class I [Ferrantini *et.al.*, 2007].

IFNα resistance has been investigated and reported for a long time. In most cases, the resistance was correlated with the signal transduction pathways activated by IFNα. Briefly, IFNα activates the tyr kinase Jak-1 and Tyk-2 after binding to its surface receptor. Then, the downstream pathways of Jak-1 and Tyk-2 which involve the signal transducer and transactivator (STAT) proteins, phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) were activated (Fig. 2.4.6) [Caraglia *et.al.*, 2005]. The suggested resistance mechanisms include defective Jak-Stat activation [Shang *et.al.*, 2007], expression of COX-2 via STAT1 activation [Lee *et.al.*, 2006], and lack of STAT1 expression [Landolfo *et.al.*, 2000].

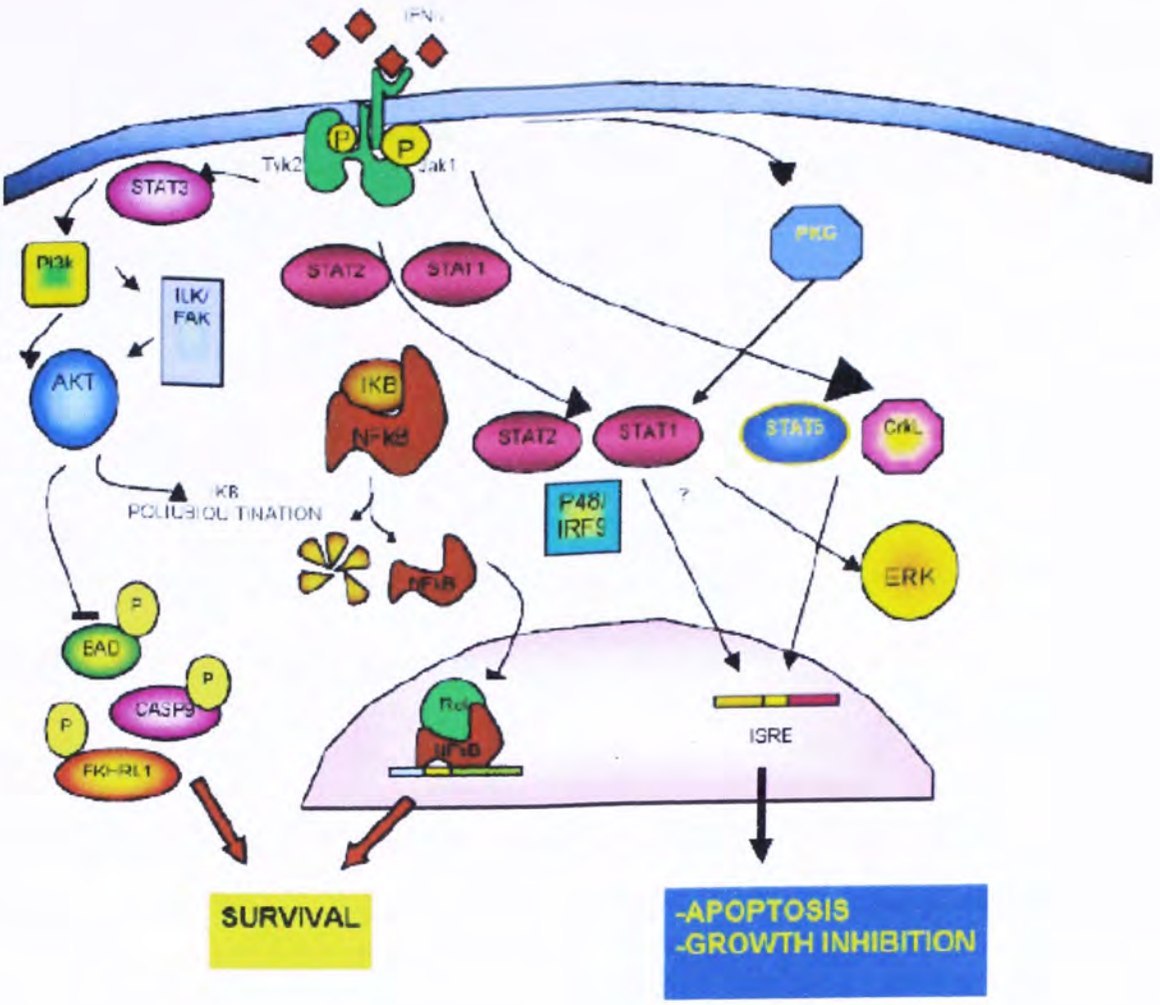


Fig. 2.4.6. Signal transduction pathway activated by IFNα [Caraglia *et.al.*, 2005].

2.4.4.2 Interferon gamma (IFNγ)

IFNγ is a type II interferon and is structurally different from IFNα. Similar to IFNα, IFNγ also signals through Jak-Stat pathway. However, it binds to a different receptor called IFNγ- receptor (IFNGR) and secreted from limited cell types including CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, NK cells, B cells and professional antigen-presenting cells (APCs). IFNGR is constructed by two ligand-binding IFNGR2 chains and two signal-transducing IFNGR2 chains. Mostly, IFNGR2 is limited and regulated by the state of cellular differentiation or activation [Schroder *et.al.*, 2004]. Therefore, the cellular

level of IFNGR2 may be one of the key targets conferring chemotherapeutic resistance of IFNγ. Likewise, alteration in Jak-Stat signaling pathway may also contribute to chemotherapeutic resistance of IFNγ. Yet, few reports have discussed about its resistance in cancer therapy.

2.4.5 Methotrexate

Antifolates such as aminopterin and methotrexate were the first antimetabolites used clinically for more than 50 years (Fig. 2.4.7). It was used to treat only a narrow spectrum of cancers such as acute lymphoblastic leukemia, osteosarcoma and breast cancer. Methotrexate is now the only antifolate widely used in cancer therapy. The cellular target of methotrexate is the enzyme dihydrofolate reductase (DHFR) involved in folate metabolism. Inhibition of DHFR results in depletion of folate cofactor which is a key component of purines, thymidylate and amino acids. In addition, polyglutamation of MTX inhibits the action of folate-dependent enzymes, thymidylate synthase, in thymidylate synthesis and glycinamide ribonucleotide formyltransferase in purine de novo synthesis. Since MTX has a polar glutamate side chain, it cannot pass through the cell membrane. Instead, it enters the cell either via a carrier-mediated system, the reduced folate carrier (RFC) or by membrane-associated folate receptors [Jansen and Pieters, 1998, McGuire, 2003].

Like other chemotherapeutic agents, resistance was also observed in MTX. It is reported that resistance may be due to decreased MTX uptake as a result of decreased RFC expression [Jansen and Pieters, 1998]. Besides, alteration of DHFR either in the expression or the affinity for MTX also contributes for the resistance. In

addition, suspected resistance determinants include low rates of thymidylate synthesis, impaired polyglutamylation, upregulation of the folate-cofactor and the availability of thymidylate and purines [Zhao and Goldman, 2003].

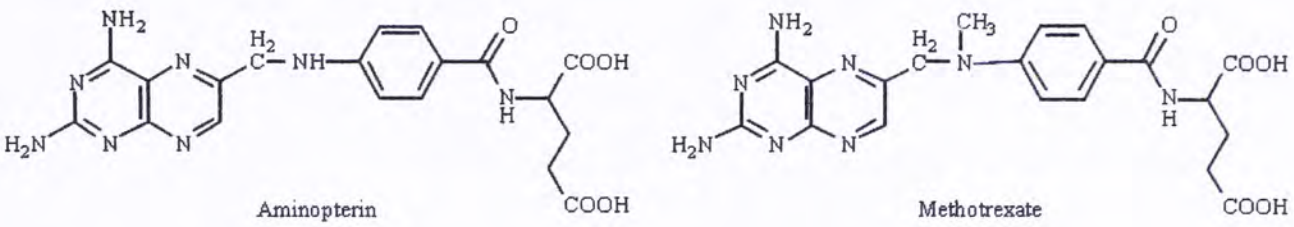


Fig. 2.4.7. The structure of aminopterin and methotrexate [McGuire, 2003].

2.4.6 Vincristine

Vincristine is a natural alkaloid present in the leaf of the periwinkle plant *Catharanthus roseus* (L.) G. It has been used for anticancer therapy such as acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma for more than 30 years. The molecular structure of vincristine is composed of a vindoline linked to a catharanthine (Fig.2.4.8). Vincristine is a mitotic inhibitor that interferes with microtubules function. At high concentration, it binds to tubulin to prevent mitotic spindle microtubules from polymerization resulting in microtubule disruption and inhibition of mitosis. At low concentration, however, it stabilizes the dynamics of microtubules and inhibits the segregation of chromosomes resulting in metaphase arrest and inhibition of mitosis [Gidding *et.al.*, 1999].

In vitro vincristine resistance has been investigated for a certain period of time. Among all the resistance mechanisms, overexpression of MDR protein such as P-glycoprotein [Huang *et.al.*, 2008, Ohtani *et.al.*, 2007], multidrug resistance-associated protein (MRP) 1 [Grant *et.al.*, 1994, Akan *et.al.*, 2005] and lung resistance protein [Scheper *et.al.*, 1993] was extensively reported. Besides, scientists also reported that vincristine resistance may also be related to the changes in tubulin isoforms that leads to a decreased binding affinity [Houghton *et.al.*, 1985], changes in the level of polymerized tubulin [Minotti *et.al.*, 1991], changes in the stability of microtubules [Cabral and Barlow, 1989], induction of HIF-1 alpha [Hussein *et.al.*, 2006], etc.

In most cases, HepG2 cells and A431 cells after hypoxia/reoxygenation treatment were found to be resistant towards the above mentioned drugs, except for camptothecin and vincristine in A431 cells. The proposed resistance mechanism of the mentioned drug in hypoxia/reoxygenation treated cells still need to be further investigated in order to confirm the exact resistance mechanism behind. Works such as examining the expressions of genes or proteins suspected to deal with resistance, knocking down or overexpressing the target genes and investigating the activities of various drug transporters can be done in the future.

Chapter 3

The Resistance Mechanism of Doxorubicin in A431 Cells

3.1 Introduction

Taking advantage of the success in genomics and proteomics researches, the treatment of cancer has been developed extensively in recent years. In the past, surgery and chemotherapy were the only ways of treating cancer. However, there are more kinds of treatment can be adopted to cure cancer nowadays such as radiation therapy, immunotherapy, hormonal therapy, monoclonal antibody therapy, etc.

3.1.1 Chemotherapeutic resistance

Chemotherapy refers to taking certain types of medicines to treat cancer. The medicines are usually worked by interfering cell cycle, disrupting DNA repair system or DNA synthesis so that cancer cells cannot grow or even die. Examples include paclitaxel which cures advanced breast cancer by interfering the breakdown of microtubule [Kumar, 1981], cisplatin which cures cervical cancer by forming DNA adducts and disrupting cell division [Tao *et.al.*, 2008], and, docetaxel which cures hormone-refractory prostate cancer by affecting the assembly of microtubule and initiating apoptosis [Caffo *et.al.*, 2008]. Although chemotherapy is so effective in treating various types of cancer, resistance always exists in certain patients or in particular circumstances. The failure of a specific type of chemotherapy may be due to poor absorption, rapid metabolism of the drug, alternations in genetic or protein expressions, reduced intracellular accumulation of the drug by efflux pump, inability of delivering the drug to the inner part of a bulky tumor or changing microenvironment of the tumor [Gottesman, 2002]. For instance, the overexpression of PDK1or Akt1 in breast cancer cell leads to resistance to paclitaxel, doxorubicin and gemcitabine [Liang *et.al.*, 2006], the presence of multidrug resistance protein (MRP) 1 and 3 contributes to the resistance towards methotrexate by reducing the

intracellular accumulation of the drug [Zeng *et.al.*, 2001], the loss of putative tumor suppressor genes EI23/PIG8 confers resistance to etoposide through aberrant p53-dependent apoptosis [Mork *et.al.*, 2007]. In order to deal with chemotherapeutic resistance, we can either search for novel drugs or modify the present treatments by evaluating resistant mechanisms behind.

3.1.2 Tumor hypoxia

The alternation in genetic or protein expressions of carcinoma cells was proposed to be affected by tumor hypoxia. Hypoxia refers to a condition in which the oxygen tension is less than 10mmHg (1.3%). Hypoxia is usually observed in most solid tumors because this kind of tumor has limited vasculature resulting in poor and fluctuating blood flow. Cells in hypoxic regions have nutrient deprivation that induces cell cycle arrest and slower proliferation rate. Moreover, they alter gene expression so as to survive. For example, the increase in the expression of dihydrofolate reductase in response to transient hypoxia [Rice *et.al.*, 1986], the loss of DNA mismatch repair genes [Kondo et.al., 2001], the up-regulation of P-glycoprotein [Comerford et.al., 2002], etc. Thus, tumors subjected to hypoxia usually exhibit drug resistance compared with the normal tumors due to the changes in the expression of gene-encoding protein.

3.1.3 Structure and function of doxorubicin

Doxorubicin, one of the well-known anthracyclines produced from streptomyces species, has been utilized clinically for treatment of cancer for more than 30 years. It is a weak base and has a planar cyclic anthraquinone nucleus attached to an amino sugar (Fig. 3.1.1) [Dorte Nielsen *et.al.*, 1996]. However, its

detailed mechanism of action is still ambiguous. It has been suggested doxorubicin exerts its cytotoxic effect by binding to nucleic acids and inhibiting DNA synthesis [Supino *et.al.*, 1977], forming free radicals which cause the destruction of DNA [Bates and Winterbourn, 1982], crosslinking of DNA [Skladanowski and Konopa, 1994], unwinding of DNA [Fornari *et.al.*, 1994], induction of DNA lesions via the inhibition of topoisomerase II [Ramachandran *et.al.*, 1993], triggering apoptosis [Skladanowski and Konopa, 1993], and, initiating growth arrest by the disruption of cyclins [Ling *et.al.*, 1996].

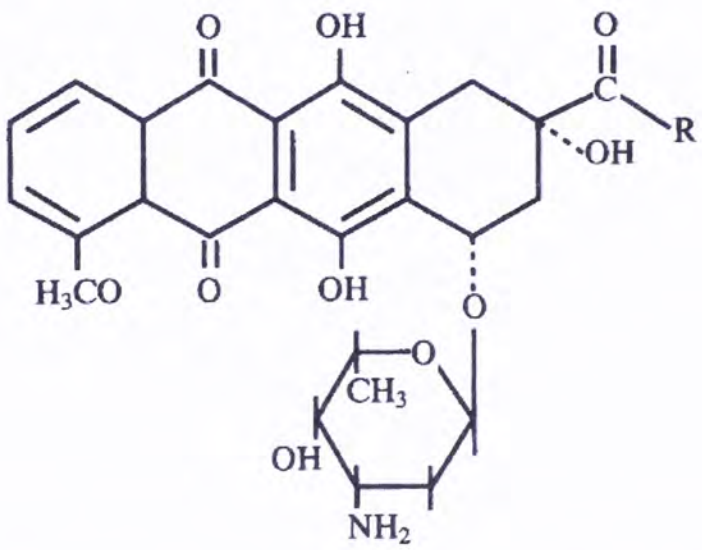


Fig 3.1.1. The structure of doxorubicin. R=CH_2OH [Dorte Nielsen *et.al.*, 1996]

3.1.4 Clinical use of doxorubicin

Although it remains unclear how doxorubicin actually exerts its cytotoxicity, it was well-known to be used in treating a broad range of cancers. However, cardiotoxicity occurs after pro-longed use of doxorubicin. Hence, a new formula of doxorubicin was discovered which was named liposomal anthracyclines. This drug was encapsulated within liposome so that it can persist in circulation and only exerts its effect in the site of neoplastic cells. Two liposomal formulations of doxorubicin

available in market are liposome-encapsulated doxorubicin-citrate complex (LED-Myocet®) and pegylated liposomal doxorubicin (PLD-Caelyx®) [Lorusso *et.al.*, 2007]. Mostly, doxorubicin is used to treat breast cancer. According to a recent report, PLD was the front-line treatment of recurrent/metastatic breast cancer [Adamo *et.al.*, 2008]. Other than breast cancer, other types of cancer such as prostate cancer [Heidenreich *et.al.*, 2004], acute myelogenous leukemia [Lu *et.al.*, 2007] and advanced neuroblastoma [Inge *et.al.*, 2004] are also treated by doxorubicin. Besides using doxorubicin solely in treating cancer, it was often administered in combination with other medicines to cure metastatic cancers. A report stated that metastatic urothelial carcinoma cured by the combination of doxorubicin, gemcitabine, paclitaxel and carboplatin, reached 56% overall response rate and a 15-month median survival [Barocas and Clark, 2008].

3.1.5 Mechanisms of doxorubicin resistance

Like other drugs, resistance also exists for doxorubicin treatment and is multifactorial. (1) Existence of classic multidrug resistance phenotype (MDR). That means the presence of P-glycoprotein (P-gp) which extrudes doxorubicin. Consequently, it decreases the intracellular accumulation of doxorubicin. (2) Non-P-gp-mediated MDR includes energy-dependent efflux pumps other than P-gp. Multidrug resistance-associated protein (MRP), Lung resistance-related protein (LRP) 56 and P95 are examples. Like P-gp, these proteins also belong to ATP-binding cassette (ABC) transporters and work by pumping drugs out of the cell. (3) Shifting of drug distribution from nuclear to cytoplasmic. (4) The overexpression of glutathione transferases (GST) confers doxorubicin resistance through the formation of nucleophilic thioether or oxidation-reduction reactions. (5) Alternation of

topoisomerase II (TOPO II) either by downregulation of TOPO II mRNA level, enhanced TOPO II protein degradation, deletion of one allele of TOPO II, mutations of TOPO II or posttranslational modification. (6) Increased DNA repair. Among all the possible mechanisms, the overexpression of P-gp is the most characterized phenomenon for the resistance of doxorubicin [Nielsen *et.al.*, 1996].

3.1.6 Structure and function of P-glycoprotein

P-glycoprotein (P-gp), with a molecular weight of 170 kD, is one of the ATP-binding cassette (ABC) transporters. There are 48 different ABC transporters which are classified into A to G based on sequence similarities. Each P-gp comprises two transmembrane domains and two ABCs (Fig. 3.1.2) and presents only in limited places such as liver, kidney and ovary. Besides, it only transports neutral and cationic hydrophobic molecules but not anionic ones. In macroscopic view, the two transmembrane domains function as a single transporter by interacting with each others' ATP sites and the major sites for the binding of chemotherapeutic molecules lie on transmembrane domains 4, 5, 6, 10, 11 and 12. They can bind various classes of drugs including Vinca alkaloids, anthracyclines, antibiotics, and other cytotoxic agents like mitomycin and Taxol [Ambudkar *et.al.*, 2003]. When one of the above molecules approaches the binding sites of P-gp, ATP hydrolysis is triggered at either one of the ATP sites and changes the shape of P-gp. In turns, the molecule is expelled from the cell [Ramachandra *et.al.*, 1998].

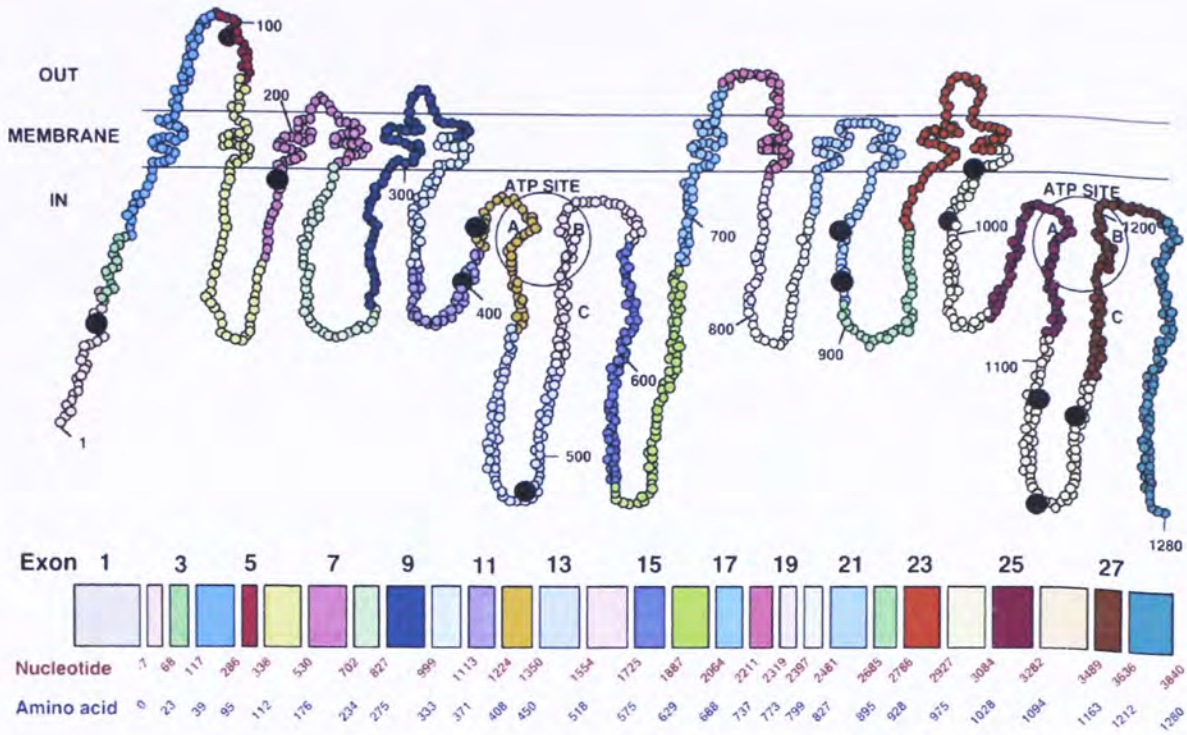


Fig. 3.1.2. The structure of P-glycoprotein. [Ambudkar *et.al.*, 2003]

P-gp is encoded by MDR1 gene which is located on chromosome 7 at q21.1 [Takara *et.al.*, 2006]. With the size of 4.6kb, MDR1 mRNA was proposed to be regulated by several transcription factors including a p53 element [Chin *et.al.*, 1992], a complex of NF-kappa B/p65 and c-Fos proteins [Ogretmen et.al., 1999], heat shock transcription factor 1 [Vilaboa *et.al.*, 2000] and a steroid xenobiotic receptor element [Synold *et.al.*, 2001].

3.1.7 Drug resistance contributed by P-glycoprotein and the solution

P-gp has been shown to confer drug resistance in cancer therapy. For example, P-gp extrudes daunorubicin in renal clear cell carcinoma [Soto-Vega *et.al.*, 2008] and mediates the efflux of imatinib mesylate in chronic myelogenous leukemia [Illmer *et.al.*, 2004]. In most cases, the overexpression of P-gp is the main cause of poor clinical result of chemotherapy. Table 3.1.1 shows some examples of P-gp

related drug resistance [Takara *et.al.*, 2006]. Moreover, upregulation of mdr1 level was found after chemotherapy (Table 3.1.2).

Table 3.1.1. Correlation of MDR1 expression with the response to chemotherapy in different types of cancer. CR: complete response [Takara *et.al.*, 2006]

Type of tumor	MDR1 expression	
	Positive	Negative
AML	27% CR	73% CR
Acute leukemia	33% Remission	74% Remission
AML	60% CR	92% CR
AML	44% CR	100% CR
AML	80% Resistant disease	64% CR
Lymphoma	55% CR	47% CR
Multiple myeloma	25% Response	75% Response
Breast cancer	7% CR	23% CR
SCLC	75% Response	86% Response
Ewing's sarcoma	17% Response	67% Response
Soft tissue sarcoma	55% CR	80% CR
Neuroblastoma	46% CR	84% CR

In order to overcome the problem of MDR caused by P-gp, scientists have designed some modulators to reverse the situation. Among all compounds, verapamil and cyclosporine are typical examples. Verapamil has been shown to reverse doxorubicin resistance in human ovarian cancer as early as 1980s [Rogan *et.al.*, 1984]. It is a calcium channel blocker and worked by inhibiting the efflux of doxorubicin molecules inside the carcinoma cells. Similarly, cyclosporine functions as a competitive substrate for the binding site of P-gp [Tamai *et.al.*, 1990] and help improving the clinical treatment in patients [Sonneveld *et.al.*, 1992].

3.1.8 Epigenetic modulation of mdr1

Recent studies focuses on numerous scientists were keen on studying the epigenetic modulation of mdr1. It has been suggested that DNA methylation regulates the expression of mdr1. In fact, genes such as cytokines [Szalmas *et.al.*, 2008] and p53 [Pogribny *et.al.*, 2002] have been found to be regulated by methylation. DNA methylation refers to the addition of a methyl group (CH_3) to the deoxycytidine with the formation of 5-deoxymethylcytidine in a CpG dinucleotide. This reaction is catalyzed by DNA methyltransferase (Fig. 3.1.3). MDR was found to be related to the alternations of methylation status of the chemotherapeutic responding genes. For example, hypermethylation at WTH3 promoter region was linked to the MDR phenotype in drug-resistant breast cancer cells [Tian *et.al.*, 2005]. Thus, the demethylating agents such as 5'-aza-2'-deoxycytidine (5-Aza-dC) may be used to re-express the silenced target genes and reverse the MDR phenotype.

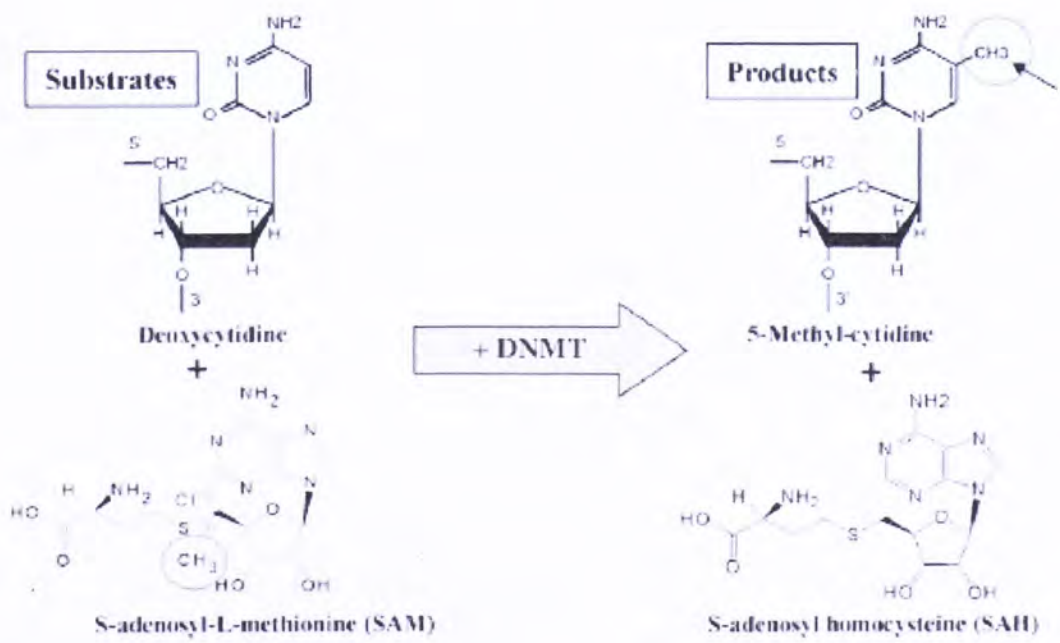


Fig. 3.1.3. A schematic diagram representing the process of DNA methylation. Arrow indicates the place of methylation. [Sulewska *et.al.*, 2007]

In this project, the sensitivity and mechanism of resistance towards doxorubicin in hypoxia/reoxygenation treated A431 cells were determined so as to provide an insight in designing a better treatment for curing doxorubicin-resistant solid tumors.

Table 3.1.2. Expression of mdr1 in clinical specimens before and after chemotherapy.

Type of tumor	Patient status	Expression of MDR1
AML	De novo	65%
	Secondary	80%
AML	De novo	18%
	Relapse/refractory	33%
Acute nonlymphoblastic leukemia	De novo	27%
	Relapse/refractory	64%
Actue leukemia	De novo	26%
	Relapse	35%
	Remission	30%
AML	De novo	57%
	Relapse	100%
CLL	Pre-treatment	14%
	Post-treatment	26%
Multiple myeloma	Pre-treatment	29%
	Post-treatment	50%
Multiple myeloma	Pre-treatment	6%
	Post-treatment	43%
Breast cancer	Pre-treatment	14%
	Post-treatment	43%
Breast cancer	Pre-treatment	9%
	Post-treatment	64%
Breast cancer	Pre-treatment	11%
	Post-treatment	30%
Ovarian cancer	Pre-treatment	15%
	Post-treatment	47%
Sacroma	Pre-treatment	40%
	Post-treatment	20%
Bladder cancer	Pre-treatment	75%
	Post-treatment	80%
CNS system cancer	Pre-treatment	18%
	Post-treatment	57%
Osteosarcoma	Primary	23%
	Metastatic	50%
Soft tissue sarcoma	Primary	18%
	Relapse	35%
Cervical cancer	Pre-treatment	35%
	Post-treatment	88%

AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CNS, central nervous system. Expression of MDR1 is calculated by dividing the MDR1-positive specimens by the total specimens. [Takara *et.al.*, 2006]

3.2 Materials and Methods

3.2.1 Cell culture

Preparation of human squamous carcinoma A431 cells can be referred to section 2.2.1. All the assays performed beyond were in air.

3.2.2 MTT assay

Please refer to section 2.2.3.

3.2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells with or without the drug treatment by lysing the cells with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). After the cells were lysed by pipetting up and down several times, RNA was extracted with 1-bromo-3-chloropropane (BCP) (Molecular Research Center, Cincinnati, OH, USA). Followed by precipitating RNA with isopropanol, the RNA pellet was washed once with 75% ethanol treated with diethyl cyanophosphonate (DEPC), air dried and dissolved in DEPC treated water by incubating at 55-60^{o}C for 10 minutes. To assess the integrity of the total RNA extracted, an aliquot of the RNA sample was run on a 1% denaturing agarose gel stained with ethidium bromide (EtBr). A sharp and clear 18S and 28S rRNA bands represented an intact total RNA. After confirming the integrity of RNA, its concentration was measured by reading the absorbance at 260nm and 1.2μg of the total RNA was used to perform the reverse transcription. To begin with, total RNA was incubated with 0.3μg oligo dT (Invitrogen) for 5 minutes

at 70°C and then kept on ice for 2-3 minutes. Then, the first cDNA was synthesized by using M-MLV reverse transcriptase (Promega, Madison, WI, USA) in 1X first strand RT buffer at 42°C for 60 minutes. The cDNA generated was used as a template for PCR amplification of the genes of interest. Primer sequences for human mdr1 and β-actin genes were as follows respectively: for mdr1, 5'GAATCTGGAGGAAGACATGACC-3' (forward primer) and 5'TCCAATTTTGTCACCAATTCC-3' (reverse primer); for β-actin, 5'-GCGGGAAATCGTGCGTGACATT-3' (forward primer) and 5'-GATGGAGTTGAAGGTAGTTTCGTG-3' (reverse primer). PCR amplification of the above genes were performed in a 20μl mixture containing 100nM corresponding gene specific primers, 1X PCR buffer, 2mM $MgCl_2$, 200nM dNTP and 1.25U Taq DNA polymerase (Promega) with the following conditions: for mdr1, initial denaturation at 94°C for 2 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 90 seconds; and final extension at 72°C for 7 minutes; for β-actin, initial denaturation at 94°C for 2 minutes; 25 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 40 seconds; and final extension at 70°C for 5 minutes. The PCR products were electrophoresed on a 1.2% agarose gel stained with ethidium bromide and observed with an ultraviolet transilluminator. The band intensity was analyzed with Image J 1.40g.

3.2.4 Western blot analysis

Cells with or without the drug treatment were washed 3 times with cold PBS containing 1mM sodium ortho-vanadate and the remaining solution was then

completely removed. Afterwards, the cells were lysed with 30-50µl lysis buffer consisting of 0.9X PBS, 21µg/ml Aproptinin, 0.5µg/ml Leupeptin, 4.9mM $MgCl_2$, 1mM sodium ortho-vanadate, 1% Triton X-100 and 1mM phenylmethanesulfonyl fluoride (PMSF) by scraping with a cell lifter. The cell lysate was then kept on ice. After all the cell lysates were collected, they were centrifuged at 14000rpm at 4°C for 7 minutes and the supernatant was then measured and transferred to a new 1.5ml microfuge tubes. Protein quantification was performed by transferring 0.5µl supernatant of each sample in triplicate to a 96-well plate followed by Protein Assay using BCA Protein Assay Reagent (Pierce). Equal volume of 2X sodium dodecyl sulphate (SDS) sample buffer was added to the supernatant and boiled for 10 minutes so as to denature the protein extracted. Afterwards, 15-25µg of protein samples were resolved in 8%, 12% or 15% SDS-polyacrylamide gel according to the size of the target proteins at 200V for 45 minutes and then transferred onto an Immobilon-P membrane (Millipore, USA) soaked with E-blot transfer buffer at 15V for 50 minutes. The membrane was incubated with 5% non-fat milk at 4°C overnight. In the following day, the membrane was washed with PBS-T (1X PBS with 0.1% Tween 20) twice and then incubated with primary antibody at room temperature for 2 hours. The membrane was washed with PBS-T twice again and then probed with the corresponding secondary antibody conjugated with horseradish peroxidase (Zymed, USA) in 1:10000 dilution for 1-2 hours. The membrane was washed with PBS-T twice again and the signals were developed by using ECL Western Blotting Detection Reagents (Amersham Biosciences, UK). Finally, the signals were visualized on an X-ray film (Fuji Photo Film, Japan). The band intensity was analyzed with Image J 1.40g.

3.2.5 Doxorubicin efflux assay

Since doxorubicin has intrinsic fluorescent property, the intracellular doxorubicin accumulation was determined by flow cytometric analysis. In brief, A431 cells were seeded with a density of $2x10^5$ on 35-mm dishes for 2 days. Before the addition of doxorubicin, cells were pretreated with 50μM verapamil for 2 hours. Afterwards, the cells were treated with 2μM doxorubicin at 37°C for 4 hours to allow rapid influx of drug. Cells were then harvested by trypsinization, washed once with 1X PBS and resuspended in 1X PBS. The fluorescent intensity of doxorubicin was measured at an excitation wavelength of 585nm (PE) by a FACSCanto flow cytometer (Becton Dickinson, Mountain View, Calif). Intracellular doxorubicin accumulation was measured and expressed in fluorescence units. Data collected were analyzed using software WinMDI 2.8. The percentage of doxorubicin accumulation was calculated as the ratio between the normalized peak channel value of cells without the exposure to verapamil or doxorubicin and that of the cells with the above treatment.

3.2.6 Drug sensitivity of A431 cells treated with verapamil

A431 cells with a density of 1000 cells/well were seeded on 96-well plates for 24 hours and then various concentrations of doxorubicin together with 30μM verapamil were added to the cells and incubated for 72 hours. MTT assay was performed according to 3.2.2.

3.2.7 Treatment with DNA methyltransferase inhibitor

A431 cells were seeded with a density of $2x10^5$ on 60-mm dishes for 2 days. Cells were then treated with 1μM, 5μM or 10μM 5'-aza-2'-deoxycytidine (5-Aza-dC) (Calbiochem, USA), a DNA methyltransferase inhibitor, for 24 hours. Total RNA was then isolated for RT-PCR (protocol refers to 3.2.3).

3.2.8 Drug sensitivity of A431 cells treated with 5-Aza-dC

A431 cells with a density of 1000 cells/well were seeded on 96-well plates for 24 hours and then various concentrations of doxorubicin together with 5μM 5-Aza-dC were added to the cells and incubated for 72 hours. MTT assay was performed according to 3.2.2.

3.2.9 Methylation-specific PCR (MSP)

Genomic DNA from A431 cells was extracted by DNAzol reagent and denatured by 0.2M NaOH at 37°C for 10 minutes. Then, genomic DNA was subjected to bisulfite modification by incubating with 10mM hydroquinone and 3M sodium bisulfate (pH 5) at 50°C for 16 hours. The bisulfite-modified DNA was purified and desulfonated with 0.3M NaOH followed by precipitation with ethanol and elution with water. Afterwards, MSP can be carried out and all the promoter sequences and PCR conditions were shown in Table 3.2.1. An initial PCR product was created with universal primers (Pan-S and Pan-AS) followed by a second nested PCR using methylation-specific primers (MSP-S and MSP-AS) and unmethylation-specific

primers (USP-S and USP-AS). The PCR products for MSP and and USP were resolved in 2% agarose gel electrophoresis. The percentage of mdr1 promoter methylation was calculated as the percentage for the band intensity of the methylated PCR product to the sum of the band intensity of both methylated and unmethylated PCR products, i.e. MSP x 100% / (MSP+USP). The band intensity was analyzed with Image J 1.40g.

3.2.10 Bisulfite genomic DNA sequencing

The PCR product created with universal primers (Pan-S and Pan-AS) was resolved in 2% agarose gel, extracted with QiaexII gel extraction kit (Qiagen) and subcloned into pGEM-T easy vector (Promega, Madison, WI). Clones were picked up from each sample for DNA sequencing.

Table 3.2.1. Primer sequences and PCR conditions, as modified from Enokida et.al., 2004.

	Primer sequence (5'-3')	Product size (bp)	Annealing temperature, time (cycle)
Pan-S	GGAAGTTAGAATATTTTTTTTGGAAAT	223	48°C, 30s (40)
Pan-AS	ACCTCTACTTCTTTAAACTTAAAAAAACC		
MSP-S	CGAGGAATTAGTATTTAGTTAATTCGGGTCGG	95	67°C, 30s (5) → 65°C, 30s (5) → 62°C, 30s (5) → 59°C, 30s (10)
MSP-AS	ACTCAACCCACGCCCCGACG		
USP-S	TGAGGAATTAGTATTTAGTTAATTTGGGTTGG	95	
USP-AS	ACTCAACCCACACCCCAACA		

3.3 Results

3.3.1 Drug sensitivity of A431 cells to doxorubicin

Drug sensitivity of A431 cells to doxorubicin was examined by treating the cells with 10nM, 20nM, 30nM, 40nM or 50nM doxorubicin for 5 days followed by MTT assay. Fig. 3.3.1 shows that A431 cells responded to doxorubicin in a dose-dependent manner and A10HR and A20HR cells were more resistant to doxorubicin than their parental A431 cells (AP).

3.3.2 Expression profile of mdr1 and P-glycoprotein in A431 cells

Probably, the resistance to doxorubicin was due to extensive efflux of doxorubicin by P-glycoprotein (P-gp) and hence the protein level of P-gp in the above three cell lines was measured by Western blot analysis. Fig. 3.3.2B indicates that A10HR had significantly higher expression level of P-gp while A20HR had slightly higher expression level of P-gp than that of AP cells. Importantly, the expression of mdr1 was similar after 1 cycle of hypoxia/reoxygenation treatment in all the three A431 cell lines. Since P-gp is encoded by mdr1 gene, the mdr1 mRNA expression level should also be higher in both A10HR and A20HR cells than that of AP cells. From the results of RT-PCR shown in Fig. 3.3.2A, the mRNA expression level of mdr1 was found to be much higher in both A10HR and A20HR cells than that in AP cells. The results show that the resistance of mdr1 may be related to the high level expression of P-gp.

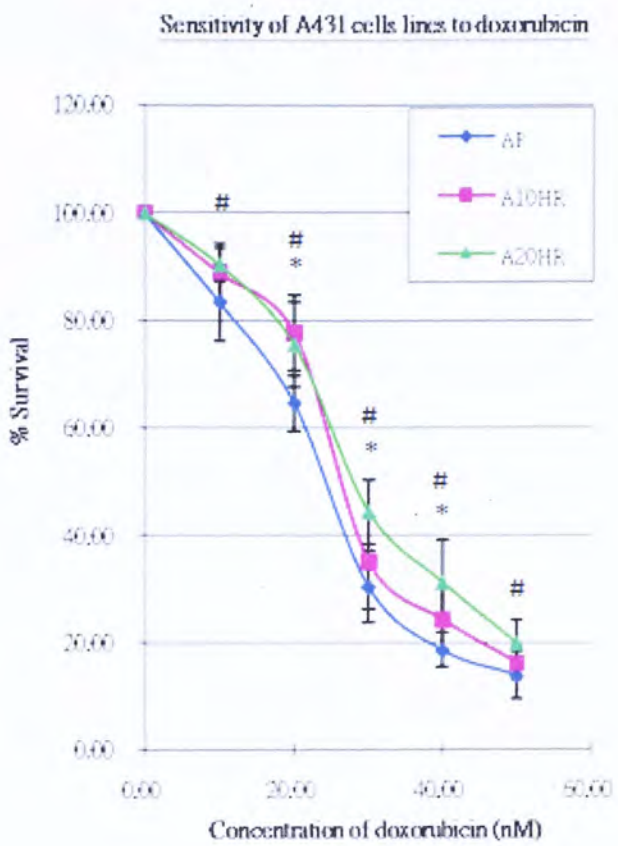
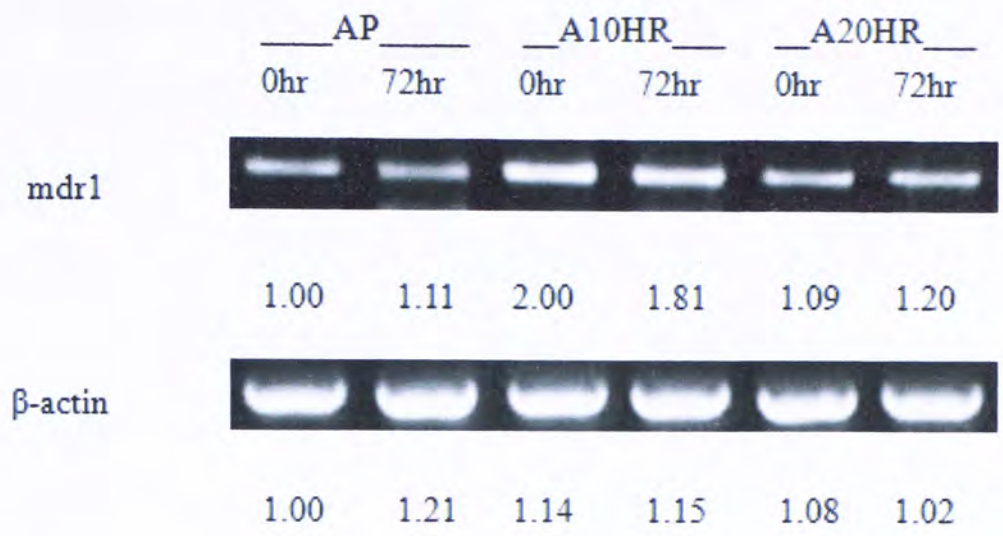


Fig. 3.3.1. The sensitivity of A431 cells to doxorubicin as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; -- ▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

*: $p < 0.05$ (between AP and A10HR); #: $p < 0.05$ (between AP and A20HR)

A.



B.

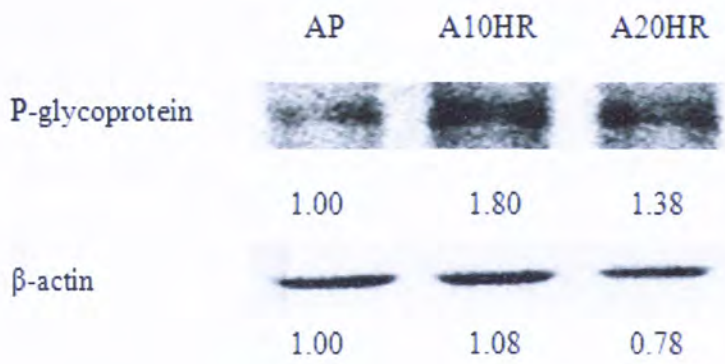
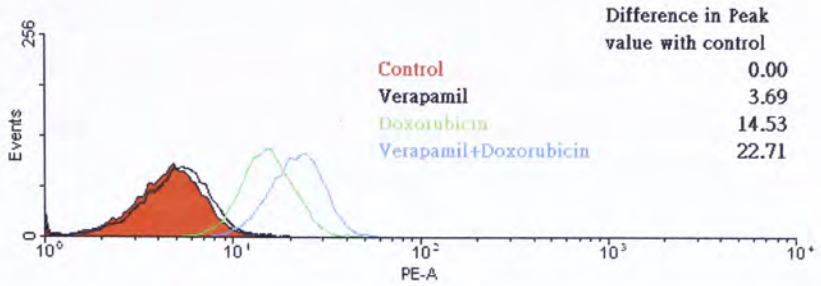


Fig 3.3.2. (A) The basal mRNA expression level of mdr1 and (B) the basal protein expression level of P-glycoprotein in A431 cells. 72HR referred to the cells treated with 72 hours hypoxia followed by 24 hours reoxygenation. The band intensity was analyzed with Image J 1.40g and the numbers showed the relative expression of mdr1 compared to the control (AP), which was designated as 1.00.

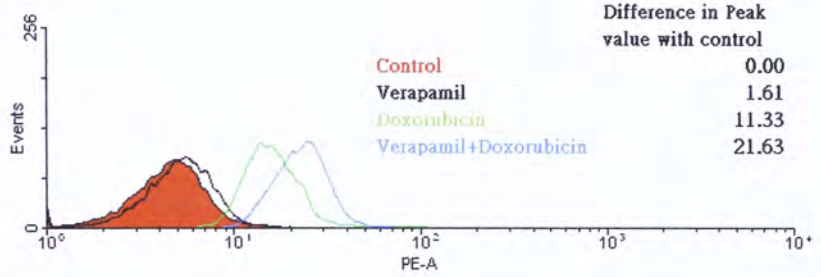
3.3.3 Dox efflux-pump activity in A431 cells

The functional activity of P-gp can be measured by flow cytometry. Generally, these energy-dependent drug efflux pumps work by removing toxic drugs taken out of the cells. Hence, the more of these efflux pumps were expressed, the more of the amount of drugs was to be released to the extracellular space, resulting in low intracellular drug accumulation. Fig. 3.3.3 shows the doxorubicin curves which represent the intracellular accumulation of doxorubicin in A431 cells in different conditions. The shifting of the curve towards the right of the x-axis indicates more doxorubicin accumulation in the cells. After the incubation of A431 cells with 2μM doxorubicin for 4 hours, more doxorubicin molecules were absorbed and retained inside AP cells compared with that of A10HR and A20HR cells. This observation can be shown by the greater shifting of the curve towards the right in AP cells. In the presence of 50μM verapamil, a calcium channel blocker that can block the activity of P-gp, fewer doxorubicin molecules were pumped out and hence more doxorubicin molecules were accumulated intracellularly, especially for the A10HR and A20HR cells which express a higher level of P-gp than that of AP. The results of the efflux activity indicate P-gp functioned normally and almost corresponded to the protein expression level of P-gp.

AP



A10HR



A20HR

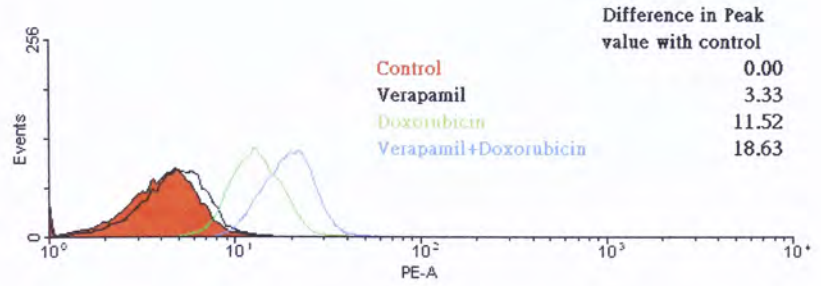


Fig. 3.3.3. Intracellular accumulation of doxorubicin in A431 cells that were treated with 2μM doxorubicin in the presence or absence of 50μM verapamil. Red area (■) represents control without exposure to doxorubicin, black line (-) represents cells exposure to verapamil, green line (-) represents cells exposure to doxorubicin, blue line (-) represents cells exposure to verapamil and doxorubicin.

3.3.4 Drug sensitivity of A431 cells in the presence of verapamil

Without the addition of verapamil, fewer AP cells survived compared with that of A10HR and A20HR upon the doxorubicin treatment because A10HR and A20HR cells possessed more P-gp that removed larger amount of doxorubicin molecules out of the cells. However, when A431 cells were co-treated with doxorubicin and verapamil, most of the P-gp was blocked and more doxorubicin molecules were trapped inside the cells. Consequently, fewer A431 cells survived compared with that of A431 cells treated with doxorubicin only (Fig. 3.3.4).

3.3.5 Expression profile of mdr1 in A431 cells in the presence of 5-Aza-dC

Previous studies had shown that the methylation status of promoters of many genes had some correlation with their expression levels. To see whether it was also true for mdr1, DNA methyltransferase inhibitor, 5-Aza-dC, was administered. When A431 cells treated with 1μM, 5μM or 10μM 5-Aza-dC, the mRNA expression of mdr1 was progressively increased. For AP cells, the relative mdr1 expression level was increased from 1.00 to 3.60. For A10HR cells, the relative mdr1 expression level was increased from 3.11 to 5.12. For A20HR cells, the relative mdr1 expression level was increased from 4.22 to 5.36. The results proved that 5-Aza-dC induced the expression of mdr1 and the expression of mdr1 may be correlated with the methylation status of mdr1 promoter in A431 cells (Fig. 3.3.5).

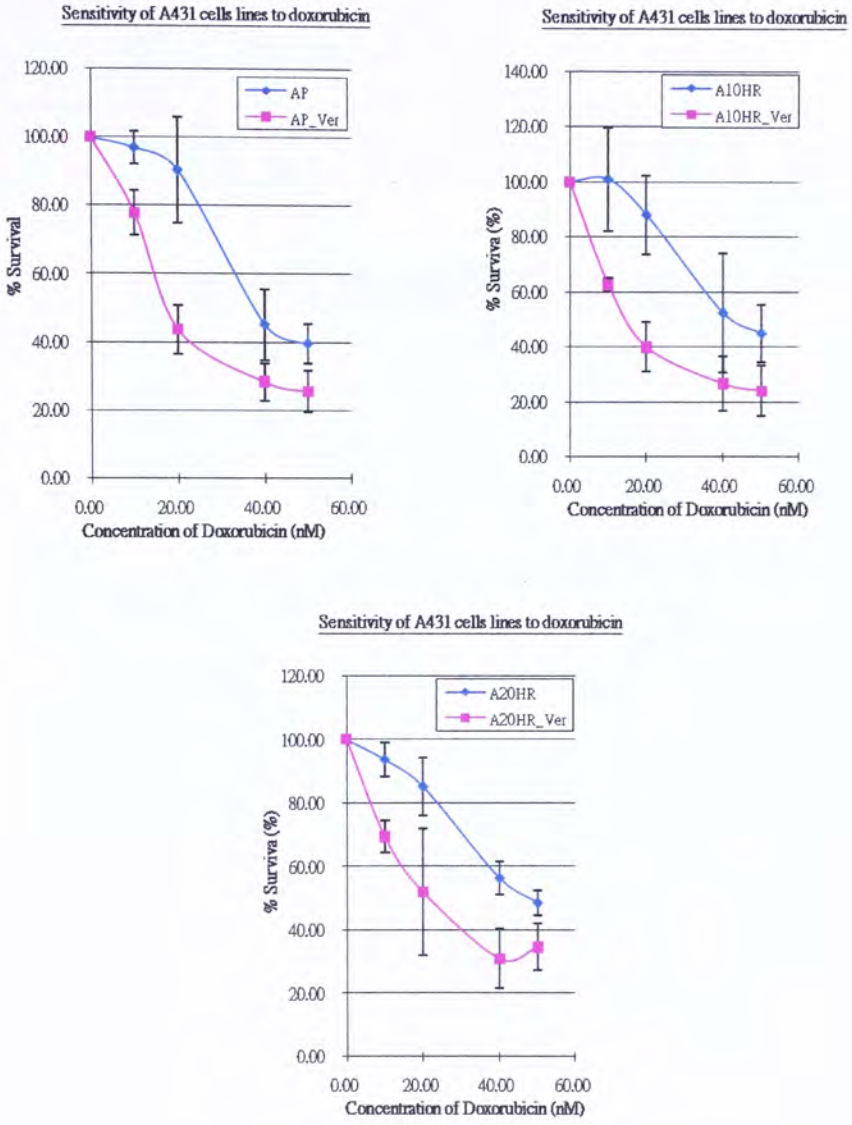


Fig. 3.3.4. The sensitivity of A431 cells to doxorubicin in the presence or absence of verapamil as measured by MTT assay. - ◆ - : cells treated with doxorubicin only; - ■ - : cells co-treated with doxorubicin and verapamil. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

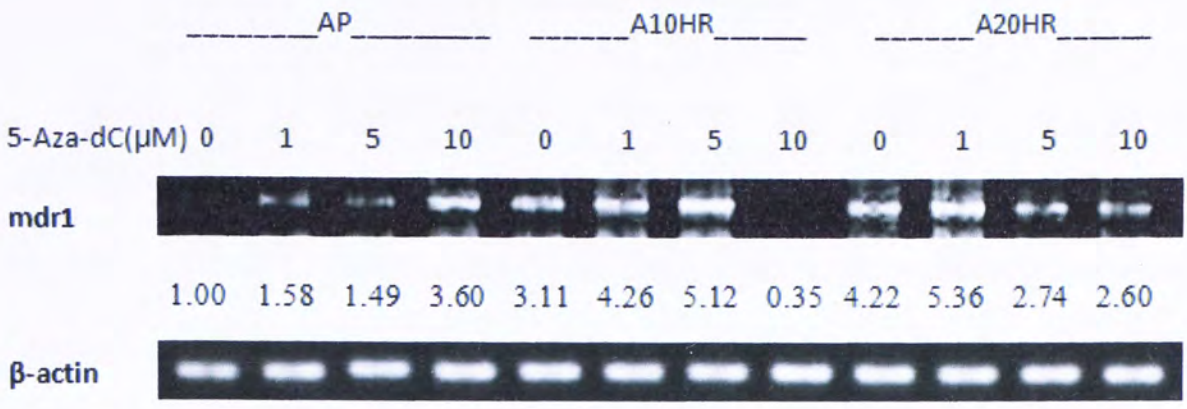


Fig. 3.3.5. The basal mRNA expression level of mdr1 in A431 cells treated with 5'-aza-2'-deoxycytidine (5-Aza-dC). The concentration of 5-Aza-dC was 0 μM, 1μM, 5μM or 10μM. The band intensity was analyzed with Image J 1.40g and the numbers showed the relative expression of mdr1 as compared to the control (AP), which is designated as 1.00.

3.3.6 Drug sensitivity of A431 cells in the presence of 5-Aza-dC

In the presence of 5-Aza-dC, more A431 cells survived when they were subjected to doxorubicin treatment, especially for AP cells (Fig. 3.3.6). It is probably due to the increased expression of mdr1 in A431 cells. According to the previous experiments, AP cells had lower expression of mdr1 than that of A10HR and A20HR cells, maybe because of their hypermethylation status of mdr1 promoter. When AP cells were treated with 5-Aza-dC, the mdr1 promoter changed from hypermethylated status to hypomethylated status resulting in increase in mdr1 mRNA templates. Consequently, more P-gp were expressed which served as the efflux pumps to remove the incoming doxorubicin molecules and the fewer AP cells were killed. Therefore, AP cells had similar percentage of survival compared with A10HR and A20HR cells when they were co-treated with 5-Aza-dC and doxorubicin.

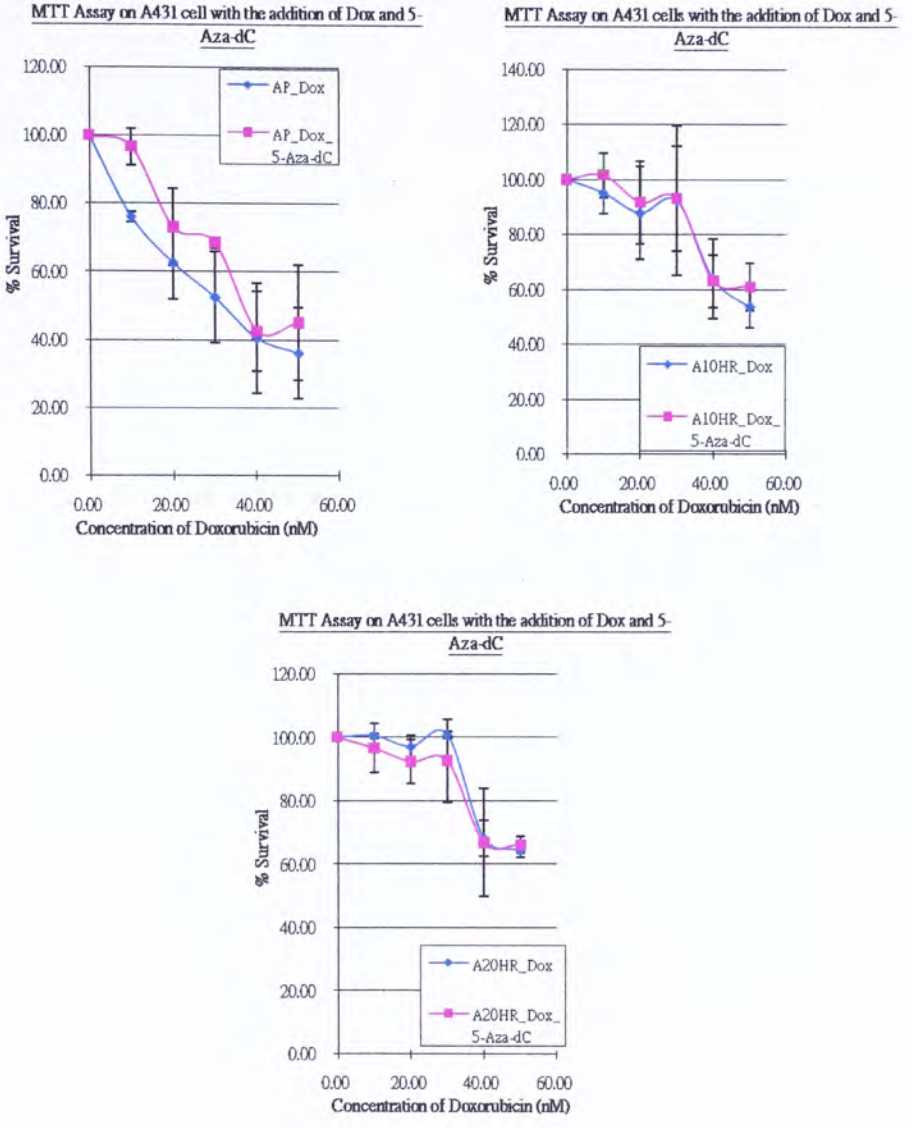


Fig. 3.3.6. The sensitivity of A431 cells to doxorubicin in the presence or absence of 5'-aza-2'-deoxycytidine (5-Aza-dC) as measured by MTT assay. - ◆ - : cells treated with doxorubicin only; - ■ - : cells co-treated with doxorubicin and 5-Aza-dC. Viability was expressed as a percentage of an untreated control.

3.3.7 Methylation status of mdr1 promoter region

To examine the methylation status of mdr1 promoter region in A431 cells, MSP was performed. It was found that a higher percentage of CpG island was methylated in AP cells (62%) compared with that of A10HR (54%) and A20HR (52%) (Fig. 3.3.7). With reference to the basal mRNA expression level of mdr1 examined previously, it may be concluded that the methylation status of mdr1 promoter region was inversely correlated with the mdr1 expression level in A431 cells.

3.3.8 Bisulfite genomic DNA sequencing of the mdr1 promoter

In order to quantify and further confirm the result of methylation-specific PCR, bisulfite genomic DNA sequencing of the mdr1 promoter in A431 cells was performed. The result revealed that AP cells had a higher percentage of methylation at the CpG island of the mdr1 promoter (82%) than A10HR cells (27%) and A20HR cells (64%). For example, complete CpG methylation at -144, -134, -110 and -105 CpG sites of mdr1 promoter in AP cells whereas all the above CpG sites were unmethylated in A10HR cells. For A20HR cells, complete CpG methylation was observed only at -144 and -110 CpG sites. The methylation status at the CpG sites of the mdr1 promoter seemed to correlate with the expression of mdr1 in A431 cells (Fig. 3.3.2). The hypermethylation of the mdr1 promoter was suggested to inactivate the expression of mdr1 but the hypomethylation of the mdr1 promoter seemed to boost the expression of mdr1.

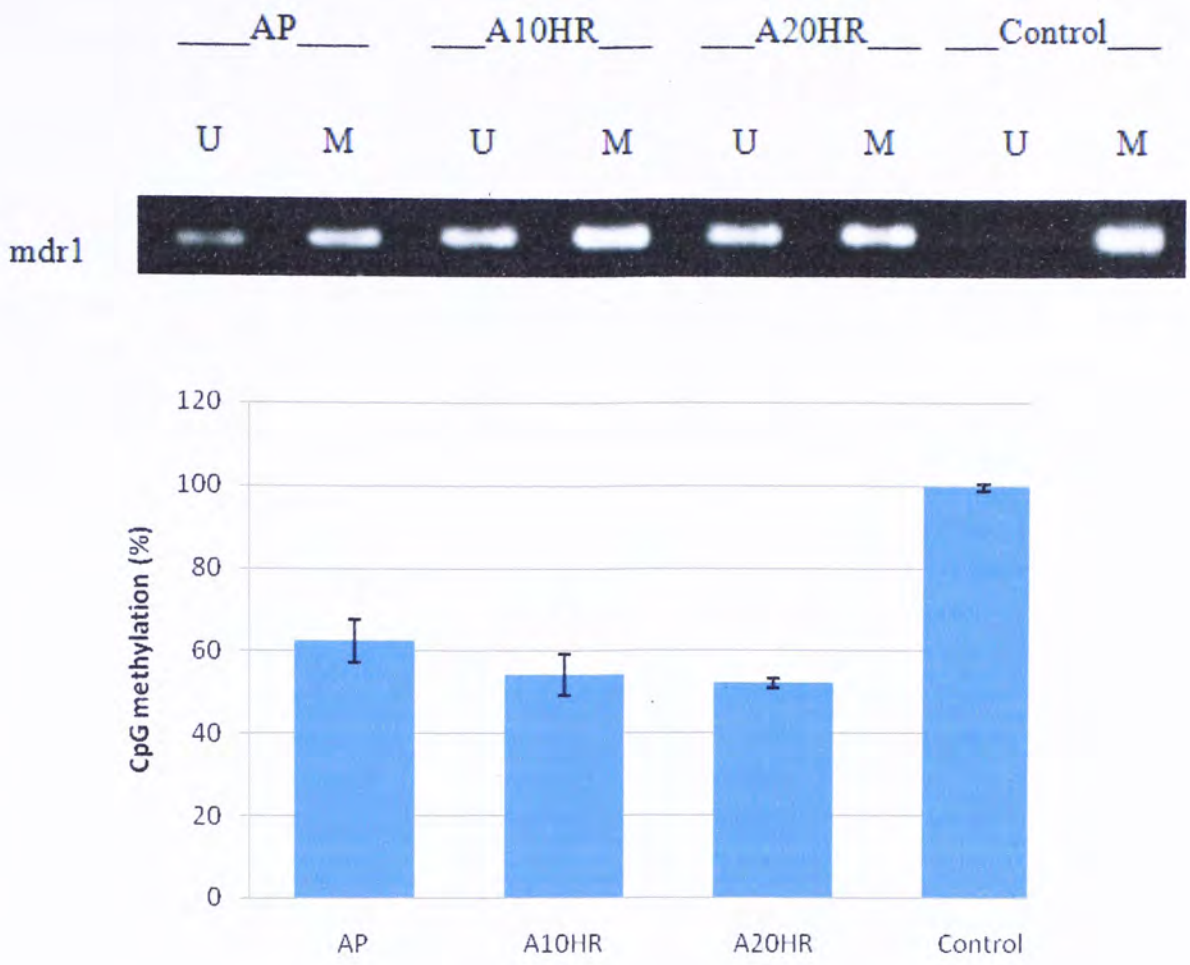
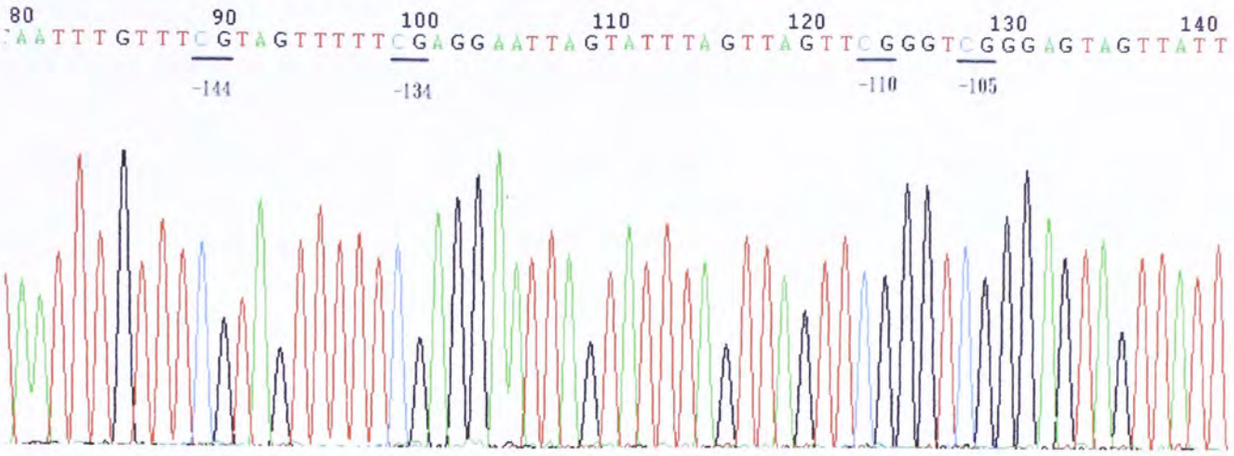
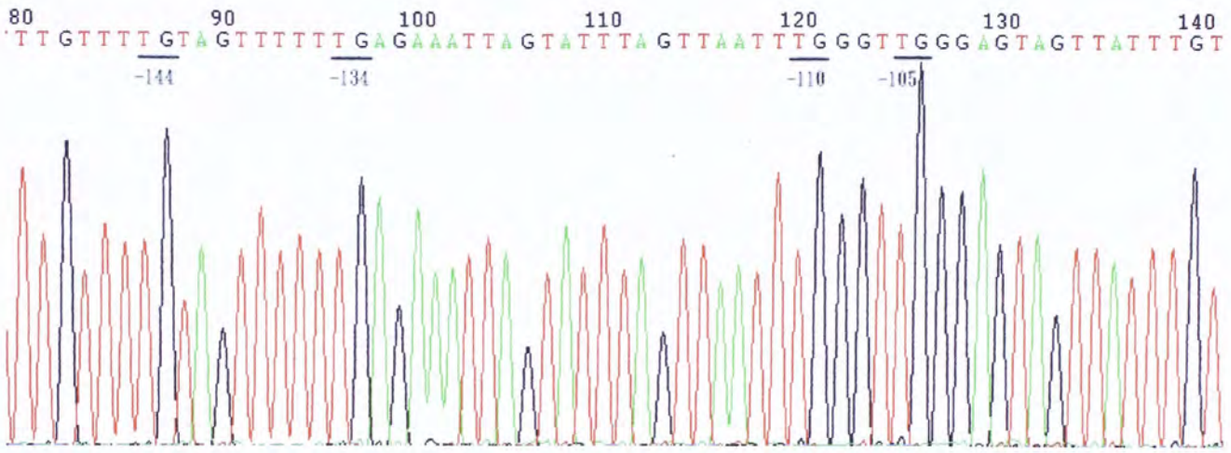


Fig. 3.3.7. Methylation status of the promoter of mdr1 gene in A431 cells by using methylation-specific PCR. U refers to the PCR product amplified by using unmethylation-specific primer; M refers to the PCR product amplified by using methylation-specific primer. Control refers to a positive control proving the success of the bisulfite modification and methylation-specific PCR. The percentage of CpG methylation of mdr1 promoter was represented by a bar chart below the bands. Error bars showed the standard deviation of at least 3 experiments.

AP



A10HR



A20HR

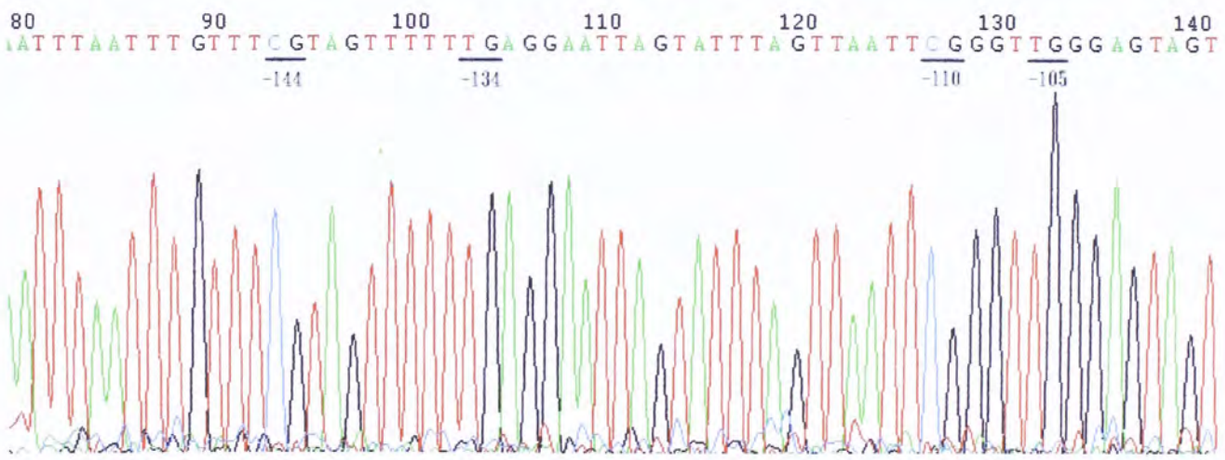


Fig. 3.3.8. Bisulfite genomic DNA sequencing of the mdr1 promoter in A431 cells. Here showed part of the promoter sequence of mdr1 with CpG sites at -144, -134, -110 and -105.

3.4 Discussion

Although doxorubicin has been revealed to confer resistance in different cancers and its resistance mechanism has been extensively studied, there is still no defined pathway regarding the resistance mechansim to doxorubicin. Moreover, there is not much research work been done on the issue of doxorubicin resistance in hypoxia/reoxygenation treated squamous carcinoma cells. Therefore, in the present study, we investigated the sensitivity and resistance mechansim of doxorubicin in hypoxia/reoxygenation treated squamous carcinoma cells A431.

From the MTT assay, A10HR and A20HR cells were more resistant to doxorubincin than that of AP cells after 5 days treatment (Fig. 3.3.1). The resistance mechanism for doxorubicin was suggested to be multifacotrial: (1) overexpression of mdr1 genes [Puhlmann *et.al.*, 2005]; (2) overexpression of apoptotic regulators [Suzuki *et.al.*, 1998]; (3) alternation of genes involved in the peroxisome proliferator-activated receptors gamma pathway [Rajkumar and Yamuna, 2008]; (4) Induction by molecules like cyclooxygenase-2 [Singh *et.al.*, 2008] and ceramide [Liu *et.al.*, 2008], etc. Among all, the overexpression of mdr1 was the most extensively investigated. Thereby, we hypothesized that the differences in sensitivity to doxorubicin was due to the differences in the expression level of mdr1.

From the results of RT-PCR (Fig. 3.3.2), the level of mdr1 mRNA in A10HR and A20HR cells was higher than that in AP cells. In addition, the protein level of mdr1 encoded P-gp in A10HR and A20HR cells was shown to be higher than that in AP cells by Western blot (Fig. 3.3.2). These results were compatible with the sensitivity of the A431 cells to doxorubicin. In cells, P-gp functions as an energy-dependent drug efflux pump to extrude doxorubicin. The higher the expression level

of P-gp, the larger the proportion of the intake doxorubicin molecules are extruded. The increased expressions of both the mdr1 gene and P-gp were suspected to be contributed by hypoxia/reoxygenation. Previous report suggested that ambient hypoxia induced mdr1 gene in a time-dependent manner [Comerford *et.al.*, 2002]. However, we found that the expression of mdr1 did not change much after one cycle of hypoxia/reoxygenation treatment.

To further confirm the above results, the efflux pump activity of A431 cells was determined (Fig. 3.3.3). Flow cytometry revealed that the efflux pump P-gp functions properly to reduce the intracellular accumulation of doxorubicin. Since A10HR and A20HR cells had more expression of P-gp, fewer doxorubicin molecules were trapped intracellularly than that of AP cells. That explained why more A10HR and A20HR cells survived upon doxorubicin treatment than that of AP cells. Verapamil, which was a calcium channel blocker, were commonly used as a modulator to reverse MDR phenotype [Wu *et.al.*, 2007]. Although this first generation reversal agent was not totally promising, it was often used clinically and the third generation modulators such as biricodar and laniquidar were developing and under clinical trials [Nobili *et.al.*, 2006]. When the A431 cells were co-treated with verapamil and doxorubicin, more doxorubicin molecules were trapped intracellularly, especially for A10HR and A20HR cells (Fig. 3.3.3 and 3.3.4). This was contributed by the blockage of P-gp by verapamil. In this case, verapamil was believed to have the ability to reverse doxorubicin resistance in human squamous carcinoma A431.

Whether a gene would be expressed or not was believed to be regulated by the methylation status of the promoter region of the gene. Hypermethylation of the promoter region of a gene was found to silence the transciption of that gene. In the

present study, the promoter of mdr1 gene in AP cells was methylated to a greater extent than that of A10HR and A20HR cells. According to some reports, a DNA methyltransferase inhibitor 5'-aza-2'-deoxycytidine (5-Aza-dC) can trigger demethylation of a gene. This phenomenon was also true for mdr1 genes. David had pointed out that the addition of 5-Aza-dC led to the hypomethylation of the promoter of mdr1 gene in MCF-7 human breast cancer cell which result in increased expression of mdr1 [David *et.al.*, 2004]. To check if the hypermethylation status of mdr1 gene in A431 cells can be reversed, A431 cells were treated with 5-Aza-dC. The results showed that 5-Aza-dC up-regulated the mRNA level of mdr1 gene in A431 cells (Fig. 3.3.5). In addition, AP cells became more resistant to doxorubicin treatment with the addition of 5-Aza-dC (Fig. 3.3.6). In order to confirm the hypothesis that the methylation of the mdr1 promoter was correlated with the expression of mdr1 promoter and the resistance to doxorubicin, methylation-specific PCR and bisulfite genomic DNA sequencing of mdr1 promoter was carried out. The results proved that hypomethylation at the CpG sites of mdr1 promoter in A10HR and A20HR cells was somehow correlated with the enhanced expression of mdr1 and conferred resistance to doxorubicin (Fig. 3.3.7 and 3.3.8).

In conclusion, doxorubicin resistance observed in hypoxia/reoxygenation treated A431 cells was probably contributed by the higher expressions of mdr1 gene and P-glycoprotein. Furthermore, hypoxia/reoxygenation treatment was suggested to trigger the demethylation of mdr1 promoter in A431 cells so as to re-express more mdr1-encoded P-glycoprotein which confers resistance to doxorubicin.

Chapter 4

The Resistance Mechanism of Cisplatin in HepG2 Cells

4.1 Introduction

Cancer is a kind of disease in which normal cells are transformed into abnormal cells by events such as mutation and rapid division without any control. There are more than 100 different types of cancer. Except leukemia, all other cancers can form solid tumors which are masses of abnormal tissue originating from organs or soft tissues and normally do not contain fluid areas and cysts.

4.1.1 Tumor hypoxia and chemotherapeutic resistance

In most solid tumors, the condition so called hypoxia usually exists. It describes a place where the oxygen tension is lower than 10mmHg (1.3%). Indeed, the existence of hypoxia is mainly due to the abnormalities in tumor vasculature. Unlike the regular, ordered vasculature of normal tissues, the tumor vasculature is irregular, tortuous, have blunt ends and with sluggish flow [Brown and Giaccia, 1998]. Since the oxygen diffusion distance in solid tumors is around 100-150 μm, any tumor cells away from this extent will be hypoxic. Apart from the low oxygen level, nutrients and growth factors are also difficult to be transported for such a long distance from the vasculature. Also, the extracellular pH becomes lower. All these affects cell proliferation, differentiation, metabolism as well as cell cycle [Yasuda, 2008]. Ultimately, it leads to the poor prognosis in solid tumors such as non-small-cell lung

cancer [Koukourakis *et.al.*, 2003], esophageal and gastric adenocarcinomas [Driessen *et.al.*, 2006], and head and neck squamous cancer [Wigfield *et.al.*, 2008].

The efficacy of a drug treatment depends at least on the distribution of the drug within the solid tumors and this is mediated by the expressions of drug efflux pumps such as P-glycoprotein and multidrug resistance-related protein (MRP) 1. In addition, the functional gene mutations or epigenetic changes that alter the expression of gene or proteins which are the targets of the drug may be another important determinant for the drug efficacy. For example, hypoxia may lead to the loss of p53-mediated apoptosis [Graeber *et.al.*, 1996], deficiency in DNA mismatch repair [Kondo *et.al.*, 2001] and upregulation of dihydrofolate reductase [Rice *et.al.*, 1986]. All these changes are suspected to be mediated by hypoxia-inducible factor-1 (HIF-1) which is induced only during hypoxia and rapidly degraded during normoxic condition [Tredan *et.al.*, 2007].

4.1.2 Cisplatin and its action mechanism

Cisplatin is a platinum-based drug responsible for curing cancers such as head and neck cancer [al-Sarraf, 1994], ovarian cancer [Alberts, 1995] and testicular cancer [Hohnloser, 1996]. It is synthesized from potassium tetrachloroplatinate (II), $K_2[PtCl_4]$ and the product is powder in yellow color (Fig. 4.1.1). Another example of

platinum-based drug is carboplatin, which was also a target drug in this project.

The action mechanism of cisplatin is mainly based on its interaction with DNA that leads to apoptosis. When cisplatin enters the bloodstream, its chloride ligands are attacked by proteins in the blood plasma such as human serum albumin. Consequently, a larger proportion of cisplatin is deactivated. The remaining intact cisplatin then enters the target cells either by direct diffusion or through Cu-transporting proteins. Inside the cell, one of the chloride ligands of the cisplatin is replaced by water and become active. The active form of the cisplatin then reacts with the guanine of DNA to form a cisplatin-DNA adducts. Consequently, the distorted DNA is recognized by DNA binding proteins that either initiate DNA damage repair or trigger apoptosis (Fig. 4.1.2) [Alderden *et.al.*, 2006]. It was found that the cytotoxicity of cisplatin was correlated with the level and persistence of cisplatin-DNA adduct.

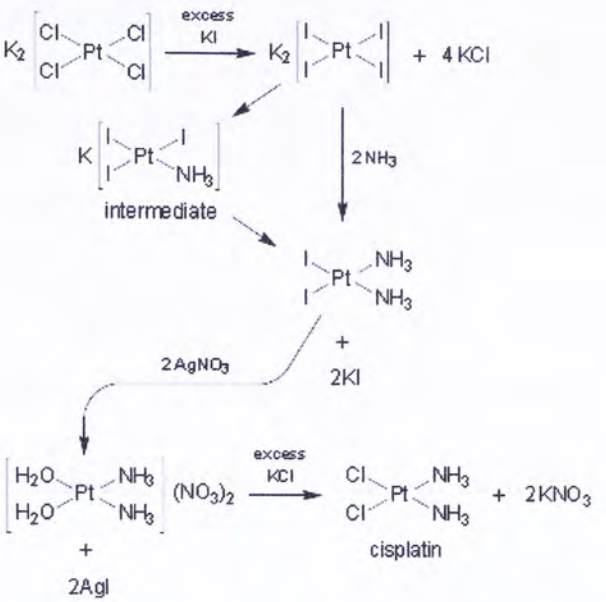


Fig. 4.1.1. A schematic diagram showing the production process of cisplatin [Alderden *et.al.*, 2006].

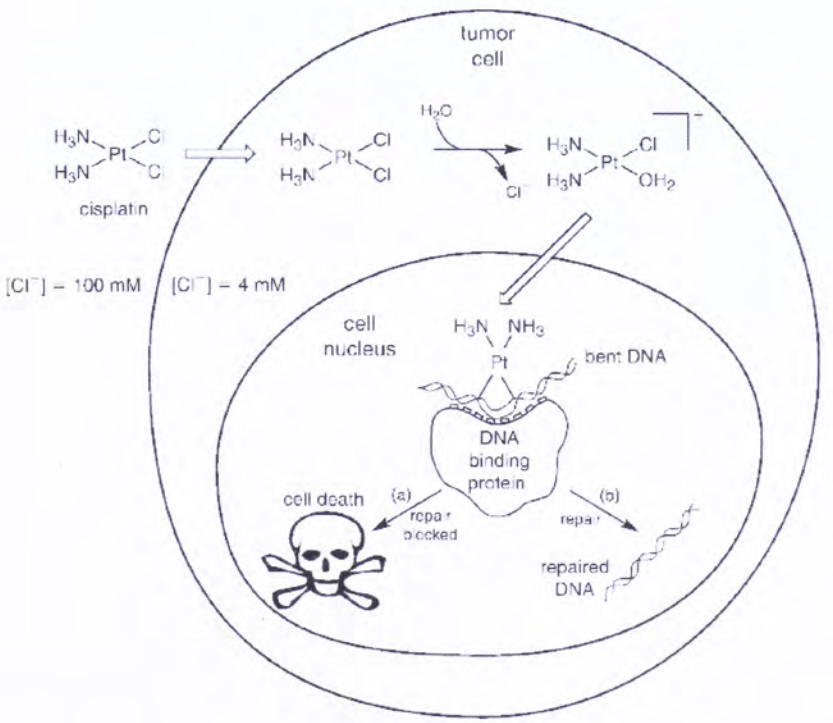


Fig. 4.1.2. The action mechanism of cisplatin [Alderden *et.al.*, 2006].

4.1.3 Mechanisms of cisplatin resistance

Generally, resistance is multifactorial. There is no exception for cisplatin. Cisplatin resistance can be acquired intrinsically or after the exposure to cisplatin. The resistance mechanism primarily deals with inhibition of apoptosis at any stages along the drug metabolic pathway. It either inhibits the formation of cisplatin-DNA adduct or signal transduction after the formation of adduct. To summarize the extensive experimental work done by scientists, the proposed mechanism of cisplatin resistance were as follows (Fig. 4.1.3): (1) reduced intracellular cisplatin accumulation by efflux pumps such as multidrug resistance-associated protein (MRP) 2; (2) enhanced inactivation of cisplatin by thiol-containing molecules like glutathione (Fig. 4.1.4); (3) enhanced repair of DNA adduct by process like nucleotide excision repair (Fig. 4.1.5); (4) deficiency of mismatch repair (MMR); (5) dysfunction of tumor suppressor gene p53; (6) overexpression of HER-2/neu and activation of the PI3-K/Akt pathway (Fig. 4.1.6); (7) attenuation of Ras and MAPK signaling pathways (Fig. 4.1.7); (8) inhibition of apoptosis such as overexpression of antiapoptotic gene Bcl-2 or suppression of caspase activity (Fig. 4.1.8) [Siddik, 2003].

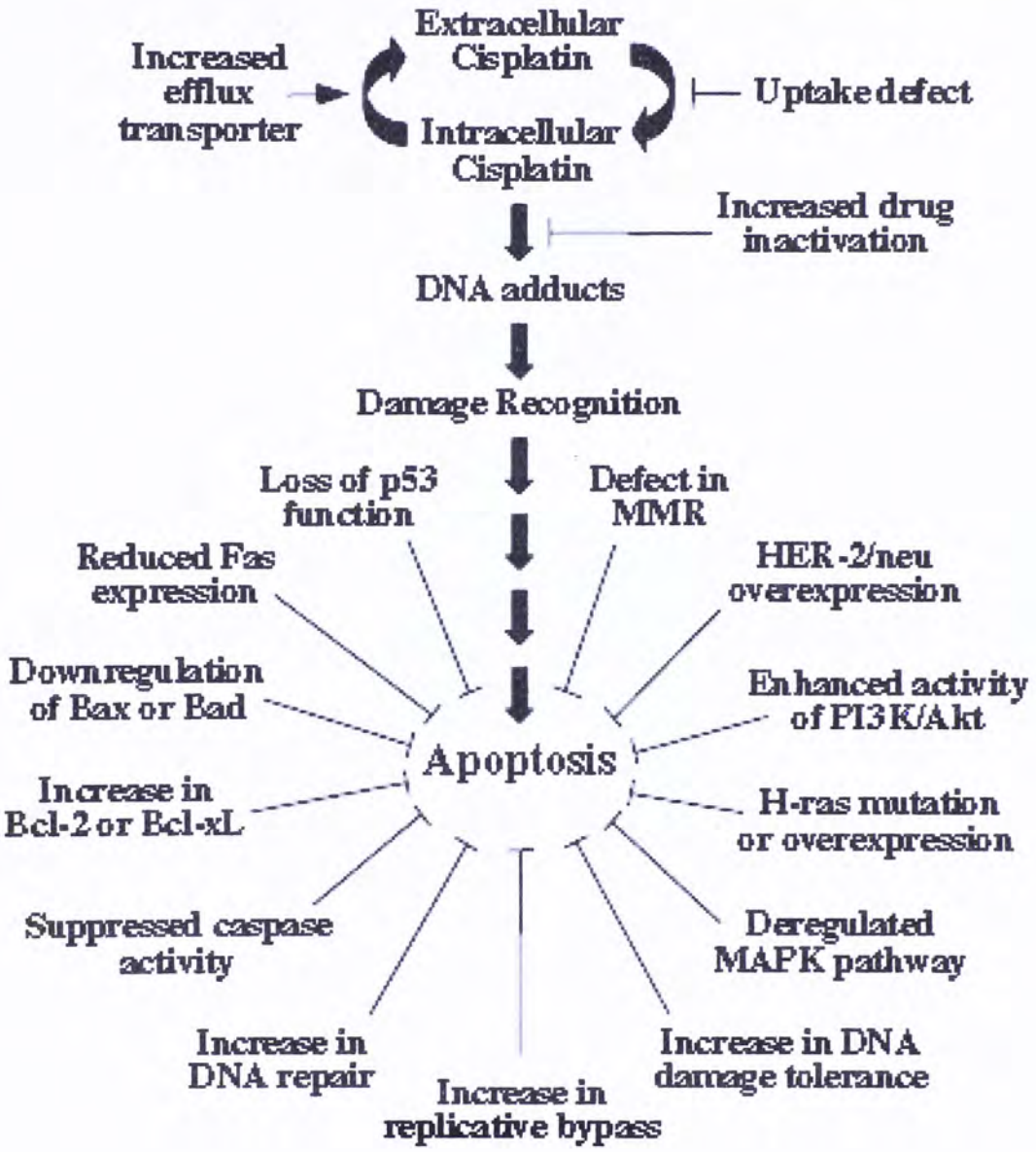


Fig. 4.1.3. Proposed mechanisms of cisplatin resistance. [Siddik, 2003]

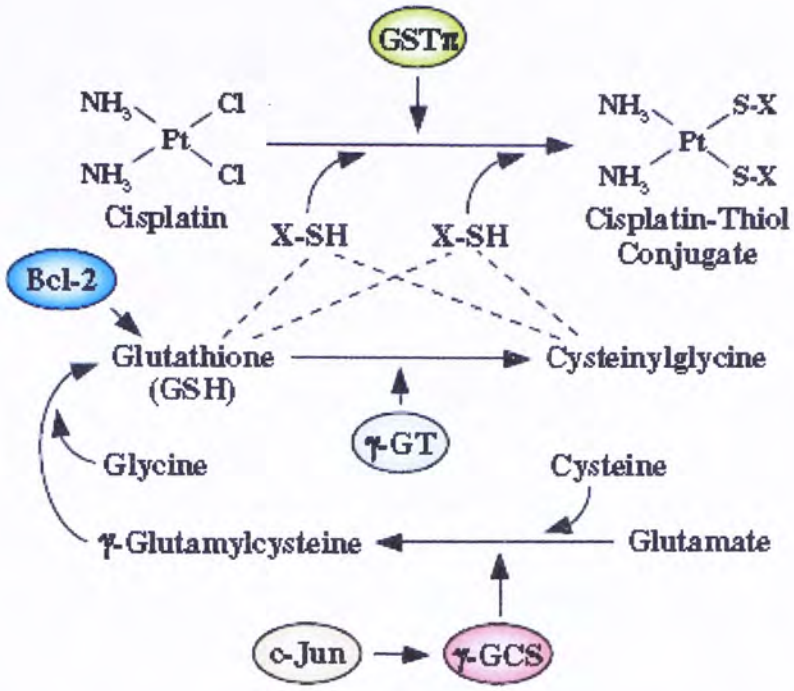


Fig. 4.1.4. Inactivation of cisplatin by thiol-containing molecules like glutathione. X-SH referred to glutathione or cysteinyl glycine. [Siddik, 2003]

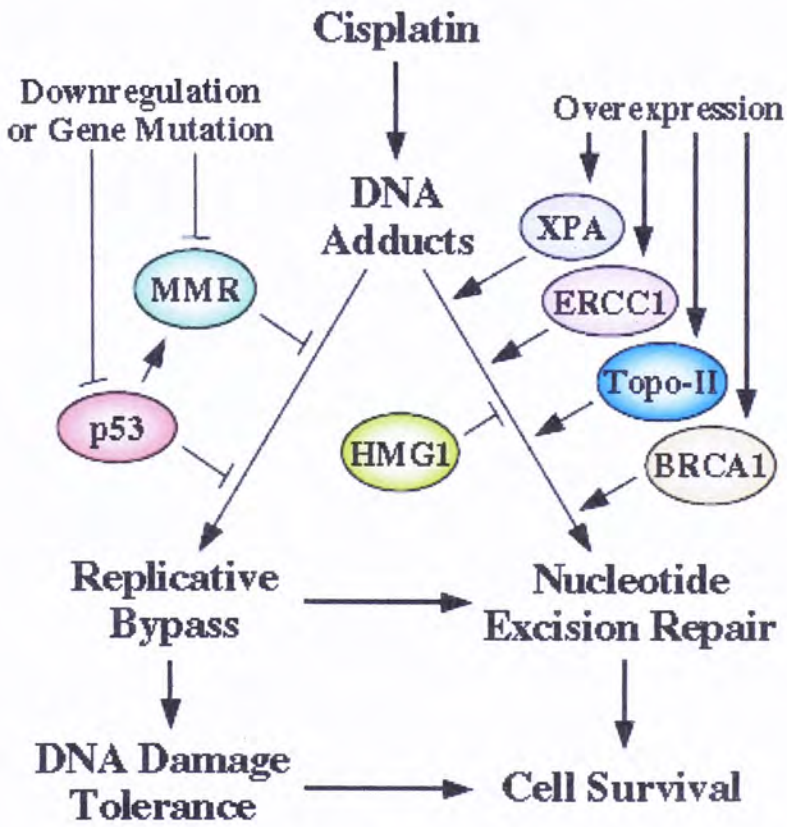


Fig. 4.1.5. How the expression of NER proteins like XPA and ERCC1 and MMR proteins affected the repair of cisplatin-induced DNA adducts. [Siddik, 2003]

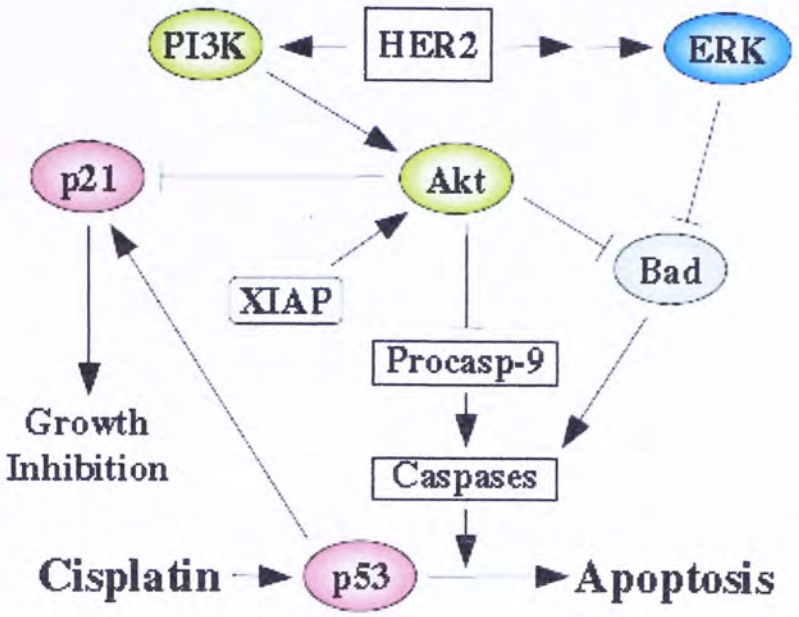


Fig.4.1.6. Overexpression of HER-2/neu and PI3-K/Akt pathway contributed to the poor response to cisplatin. [Siddik, 2003]

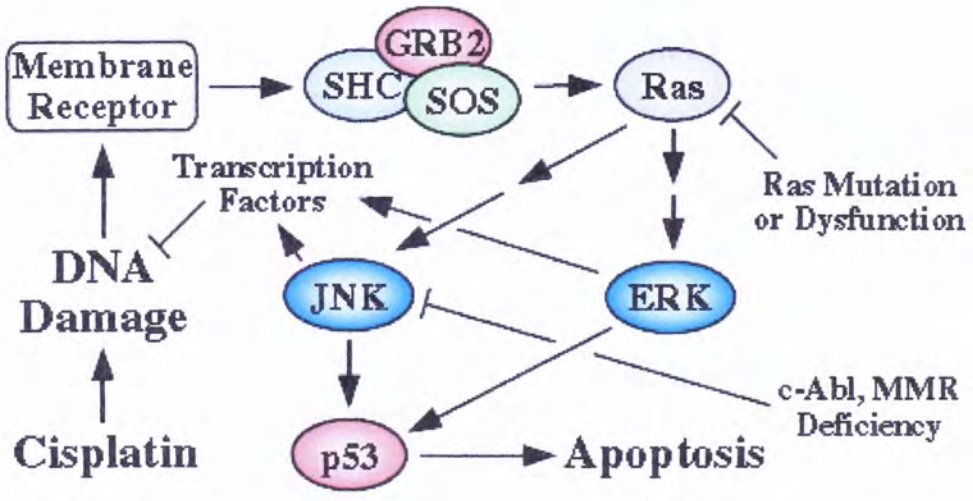


Fig. 4.1.7. Attenuation of Ras and MAPK signaling pathways in cisplatin resistance. [Siddik, 2003]

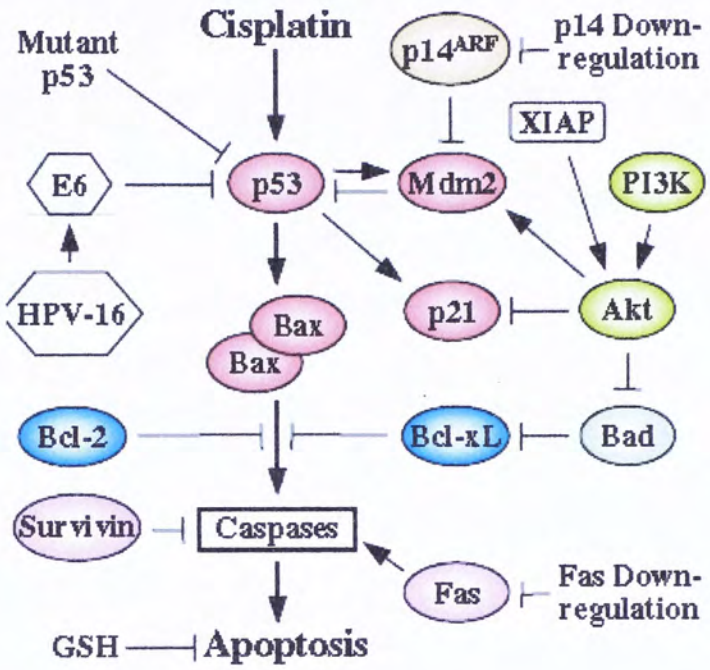


Fig.4.1.8. Inhibition and disruption of the p53-dependent apoptotic pathway contributed to cisplatin resistance. [Siddik, 2003]

4.1.4 Mismatch repair genes

Mismatch repair is a kind of DNA repair mechanism that corrects the replication errors including base-base mismatches and insertion/deletion mispairs. The mismatch repair genes in eukaryotic cells are mainly divided into two groups: MutS and MutL. MSH2, MSH3 and MSH6 belong to MutS while MLH1, MLH3, PMS1 and PMS2 belong to MutL. There are two types of MutS: MutSα (MSH2/MSH6 heterodimer) and MutSβ (MSH2/MSH3 heterodimer). MutSα, which constitutes 80-90% of MSH2, recognizes both base-base mismatches and insertion/deletion mispairs but MutSβ recognizes insertion/deletion mispairs only. On the other hand, there are three types of MutL: MutLα (MLH1/PMS2 heterodimer), MutLβ (MLH1/PMS1 heterodimer) and MutLγ (MLH1/MLH3 heterodimer). MutLα, which accounts for 90% of MLH1, interacts with MutS heterodimers and activates the downstream repair process (Fig. 4.1.9). For MutLβ and MutLγ, their involvement in repair process is uncertain [Modrich, 2006].

Defect in MMR genes will lead to microsatellite instability and defect in apoptosis which in turns contribute to drug resistance. Abundant reports suggested that the loss of MLH1 expression may be possibly responsible for cisplatin resistance [Brown *et.al.*, 1997, Strathdee *et.al.*, 1999, Plumb *et.al.*, 2000]. Other than MLH1, the mutation of MHS2 and MSH6 was linked to the tolerance of human T-cell

leukemia line to methylating agents [Levati *et.al.*, 1998].

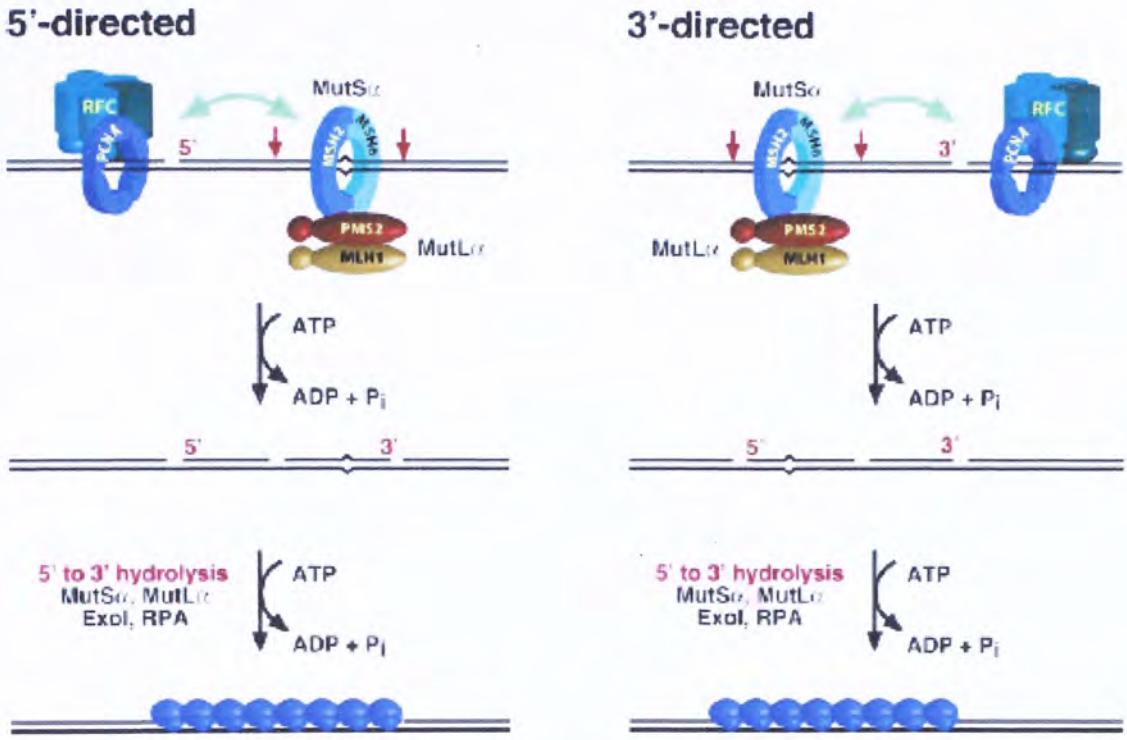


Fig. 4.1.9. The process of recognition and mismatch repair by MMR genes [Modrich, 2006].

4.1.5 Epigenome and drug resistance in cancer

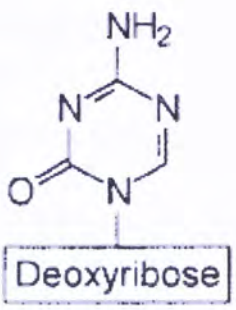
Epigenetic events have been found to be correlated with drug resistance in cancer recently. It is done by altering the gene expression without changing the DNA coding sequence that is inheritable through cell division. There are two types of epigenetic events: DNA methylation/demethylation and histone acetylation/deacetylation. DNA methylation is controlled by DNA methyltransferase

(DNMT). It was reported that hypermethylation of CpG islands, which are primarily found in the promoter region of a gene, was associated with the transcriptional silencing of the gene. In fact, each type of human cancer has its own pattern of gene hypermethylation that makes it resistance to chemotherapy. In such cases, the levels of tumor suppressor genes were usually downregulated by hypermethylation of the genes' promoter. The examples of silenced genes by this method include those for DNA repair (hMLH1), cell cycle control ($p14^{ARF}$) and apoptosis (caspase 8).

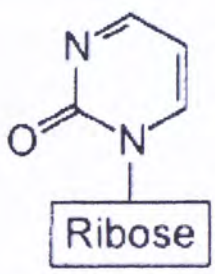
On the other hand, histone acetylation is the post-translational modification of histone proteins by histone acetyltransferases and histone deacetylases (HDAC). It is generally agreed that histone acetylation induces nucleosome remodeling and transcription activation of the gene. In contrast, histone deacetylation is mainly responsible for chromatin condensation that leads to transcription repression of the gene [Kopelovich *et.al.*, 2003, Suzuki *et.al.*, 2007] .

DNA methylation has an intimate relationship with histone deacetylation. Accordingly, DNMTs not only direct DNA methylation but also recruit HDACs for histone deactylation, resulting in transcriptional repression of the gene. In order to make drug-resistant cells become chemosensitive to anticancer drugs once again, target genes such as tumor suppressors and apoptosis-related genes must be re-expressed by the use of inhibitors of DNMT and HDAC inhibitors.

5-aza-2'-deoxycytidine (5-Aza-dC) and zebularine are examples of DNMT inhibitors (Fig. 4.1.10). These inhibitors trigger the hypomethylation of the gene and let the gene re-express. For HDAC inhibitors, trichostatin A and suberoylanilide hydroxamic acid (SAHA) are clinically used to cause hyperacetylation of histone proteins and re-activate the gene expression (Fig. 4.1.11).

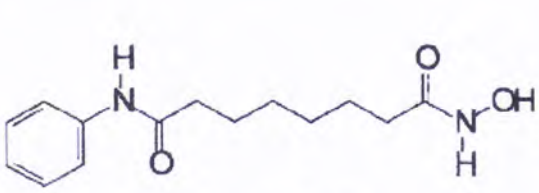


5-Aza-2'-deoxycytidine

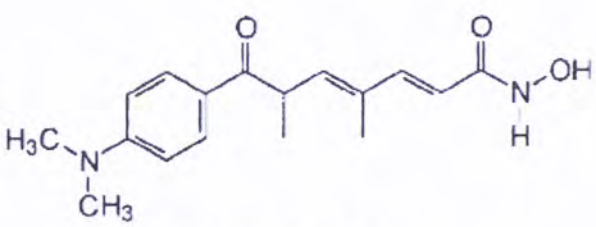


Zebularine

Fig. 4.1.10. Structures of two DNMT inhibitors. [Kopelovich *et.al.*, 2003]



Suberoylanilide Hydroxamic Acid



Trichostatin A

Fig. 4.1.11. Structures of HDAC inhibitors. [Kopelovich *et.al.*, 2003]

Epigenetic therapy has been shown to be a promising therapy in certain cancers such as resensitization of drug-resistant ovarian cancer cell lines to cisplatin by zebularine [Balch *et.al.*,2005] and reversal of drug resistance in human tumor xenografts by 5-Aza-dC [Plumb *et.al.*, 2000]. Nonetheless, problems such as non-selectable gene activation and progressive silencing of demethylated promoter impede the development of epigenetic therapy. Therefore, tremendous work remains to be done so as to achieve successful epigenetic therapy against cancer.

Although cisplatin resistance has been observed in different types of cancers both in vitro and in vivo, few reports correlate cisplatin resistance with hypoxia/reoxygenation and epigenome. In this part of the project, the resistance mechanism of cisplatin developed in hypoxia/reoxygenation treated HepG2 cells and its relationship with epigenome were investigated.

4.2 Materials and Methods

4.2.1 Cell culture

Preparation of human hepatocellular carcinoma HepG2 can be referred to 2.2.1. All the assays performed beyond were in air.

4.2.2 MTT assay

Please refer to section 2.2.3.

4.2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells with or without treatment by lysing the cells with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). After the cells were lysed by pipetting up and down several times, RNA was extracted with 1-bromo-3-chloropropane (BCP) (Molecular Research Center, Cincinnati, OH, USA). Followed by precipitating RNA with isopropanol, the RNA pellet was washed once with 75% ethanol treated with diethyl cyanophosphonate (DEPC) , air dried and dissolved in DEPC treated water by incubating at 55-60°C for 10 minutes. To assess the integrity of the total RNA extracted, an aliquot of the RNA sample was run on a 1% denaturing agarose gel stained with ethidium bromide (EtBr). A sharp and clear 18S and 28S rRNA bands visualized represented an intact total RNA. After

confirming the integrity of RNA, its concentration was measured by reading the absorbance at 260nm and 1.2μg of the total RNA was used to perform the reverse transcription. To begin with, total RNA was incubated with 0.3μg oligo dT (Invitrogen) for 5 minutes at 70°C and then kept in ice for 2-3 minutes. Then, the first cDNA was synthesized by using M-MLV reverse transcriptase (Promega, Madison, WI, USA) in 1X first strand RT buffer at 42°C for 60 minutes. The cDNA generated was used as a template for PCR amplification of interest genes. The primers for the DNA mismatch repair genes were used as reported that reported previously [Chang DK et.al. 2000] and were shown in Table 4.2.1. The PCR products were electrophoresed on a 1.2% agarose gel stained with ethidium bromide and observed with an ultraviolet transilluminator. The band intensity was analyzed with Image J 1.40g.

4.2.4 Oligonucleotide transfection

The sequences of 21-nucleotide sense and antisense RNA were as follow: PMS2 siRNA, 5'-GGUUUCAUUUCACAAUGCA(dTdT)-3' (sense) and 5'-UGCAUUGUG AAAUGAAACC(dTdT)-3' (antisense); scramble siRNA, 5'-CCUACGCCACCAAU UUCGU(dTdT) (sense) and 5'-ACGAAAUUGGUGGCGUAGG(dTdT) (antisense). PMS2 siRNA was purchased from Qiagen while the validated, non-targeting, scramble siRNA (siRNA with scrambled sequence that was not homology to any

known human gene sequences) was purchased from Tech Dragon. Transient transfection of siRNA was carried out by using Oligofectamine^{TM} reagent (purchased from Invitrogen) and followed the manufacturer's protocol. HepG2 cells with a density of 1500 cells/well were seeded on 96-well plates for 1 day until they reached 30-50% confluence. Cells were then transfected with 20nmol PMS2 siRNA or scramble siRNA per well in serum-free medium for 4 hours. Finally, the cells were cultured using DMEM supplemented with 5% FBS (Invitrogen , Carlsbad CA, USA) and incubated in a humidified incubator with 10% CO_2 at 37°C. In the following day, cisplatin with various concentrations (0μM, 0.1μM, 0.5μM, 1μM, 2μM and 5μM) were added to the cells and the incubation was for 3 days. The viability of cells were determined by MTT assay (protocol refers to 4.2.2). To check the knockdown efficiency of PMS2 by siRNA, HepG2 cells with a density of $2x10^5$ were seeded on 35mm plates for 1 day followed by transfection with 200nmol PMS2 siRNA or scramble siRNA per well in serum-free medium for 4 hours. The cells were then cultured in DMEM with 5% FBS for 1 day and the mRNA expression level of PMS2 was determined by RT-PCR (protocol refers to 4.2.3).

4.2.5 Treatment with DNA methyltransferase inhibitor

HepG2 cells were seeded with a density of $2x10^5$ on 60-mm dishes for 2 days. Cells

were then treated with 5μM or 10μM 5-aza-2'-deoxycytidine (5-Aza-dC) (Calbiochem, USA), a DNA methyltransferase inhibitor, for 24 hours. Total RNA was then isolated for RT-PCR (protocol refers to 4.2.3).

4.2.6 Drug sensitivity of HepG2 cells treated with 5-Aza-dC

HepG2 cells with a density of 1500 cells/well were seeded on 96-well plates for 24 hours and then various concentrations of cisplatin together with 5μM 5-Aza-dC were added to the cells and incubated for 72 hours. MTT assay was performed according to 4.2.2.

4.2.7 Treatment with histone deacetylase inhibitor

HepG2 cells were seeded with a density of $2x10^5$ on 60-mm dishes for 2 days. Cells were then treated with 0.2μM or 0.5μM trichostatin A (TSA) (Calbiochem, USA), a histone deacetylase inhibitor, for 24 hours. Total RNA was then isolated for RT-PCR (protocol refers to 4.2.3).

4.2.8 Drug sensitivity of HepG2 cells treated with TSA

HepG2 cells with a density of 1500 cells/well were seeded on 96-well plates for 24 hours and then various concentrations of cisplatin together with 0.5μM TSA were added to the cells and incubated for 72 hours. MTT assay was performed according to 4.2.2.

Table 4.2.1. A list of PCR primer sequences and their conditions used in this project.

Target gene	Sequences		Annealing temperature (^{o}C)	Cycle
β-actin (MMR)	Forward	5'-TACCACTGGCATCGTGATGGACTC-3'	55	25
	Reverse	5'-TCCTGCTTGCTGATCCACATCTGC-3'		
MSH6	Forward	5'-ACAAGGGGCTGGGTTAGCAAAAG-3'	55	30
	Reverse	5'-AGTCACCATTCTCTTCCGCTTTCG-3'		
MSH2	Forward	5'-TTGGCATATAAGGCTTCTCCTGGC-3'	55	30
	Reverse	5'-TGCAACCTGATTCTCCATTTCTGG-3'		
PMS2	Forward	5'-CAGGTTTCATTTCACAATGCACGC-3'	55	30
	Reverse	5'-TTACCTTCAACATCCAGCAGTGGC-3'		
MLH1	Forward	5'-TTCTCAGGTTATCGGAGCCAGCAC-3'	55	30
	Reverse	5'-CTTCGTCCCAATTCACCTCAGTGG-3'		
MSH3	Forward	5'-GATGGCATTTTCACAAGGATGGG-3'	55	30
	Reverse	5'-CTGGCGGATAATGGGTGACAAAC-3'		
PMS1	Forward	5'-GGCAACAGTTCGACTCCTTTCAAG-3'	55	30
	Reverse	5'-TTGATACCCTCCCCGTTATCTCGC-3'		

4.3 Results

4.3.1 Drug sensitivity of HepG2 cells to cisplatin

Drug sensitivity of HepG2 cells to cisplatin was examined by treating the cells with 0.1μM, 0.5μM, 1μM, 2μM or 5μM cisplatin for 5 days followed by MTT assay. It was observed that all the HepG2 cells responded to cisplatin in a dose-dependent manner (Fig. 4.3.1). The IC50 of cisplatin for G10HR and G20HR cells were about 2.25- and 2.80-fold of that for GP cells, respectively. In order to find out if there was any relationship between cisplatin resistance and the activity of the MMR genes, the mRNA expression levels in HepG2 cells were determined.

4.3.2. Expression profile of the MMR genes in HepG2 cells

The mRNA levels of six MMR genes were investigated by RT-PCR. The results in Fig. 4.3.2 showed that the mRNA levels of all the MMR genes except PMS2 were more or less the same among GP, G10HR and G20HR cells. For PMS2, the mRNA expression level was progressively down-regulated in G10HR and G20HR cells. It was hypothesized that the cisplatin resistance observed in G10HR and G20HR cells was related to the down-regulation of PMS2. Hence, PMS2 was knocked down by the use of siRNA so as to see if PMS2 played a role in cisplatin resistance in G10HR and G20HR cells.

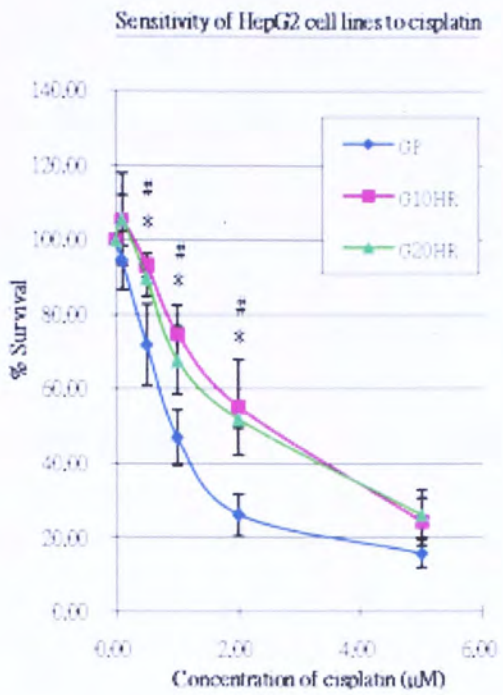


Fig. 4.3.1. The sensitivity of HepG2 cells to cisplatin as measured by MTT assay. - ◆ - : parent cells; - ■ - : conditioned cells after 10 hypoxia/reoxygenation cycles; --▲-- : conditioned cells after 20 hypoxia/reoxygenation cycles. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

*: $p < 0.05$ (between AP and A10HR); #: $p < 0.05$ (between AP and A20HR)

4.3.3 Drug sensitivity of HepG2 cells to cisplatin after the knock-down of PMS2

PMS2 was knocked down by the use of PMS2 siRNA (PMS2i). After PMS2i transfection, the mRNA level of PMS2 was found to be down-regulated to about 50% of the original expression level (Fig. 4.3.3). The results suggested the PMS2 siRNA successfully knock-down the expression of PMS2. Afterwards, the sensitivity of HepG2 cells to cisplatin after PMS2i transfection was examined (Fig. 4.3.4). Interestingly, GP cells, which possessed higher basal mRNA level of PMS2, became more resistant to cisplatin while G10HR cells, which possessed lower basal mRNA level of PMS2, responded similarly to cisplatin after PMS2i transfection. It agreed with the hypothesis that down-regulation of PMS2 increased the cisplatin resistance in HepG2 cells. However, the reason for differences in the expression level of PMS2 in the three HepG2 cells remained unsolved. One of the possible ways to regulate the expression of a gene was epigenetic regulation.

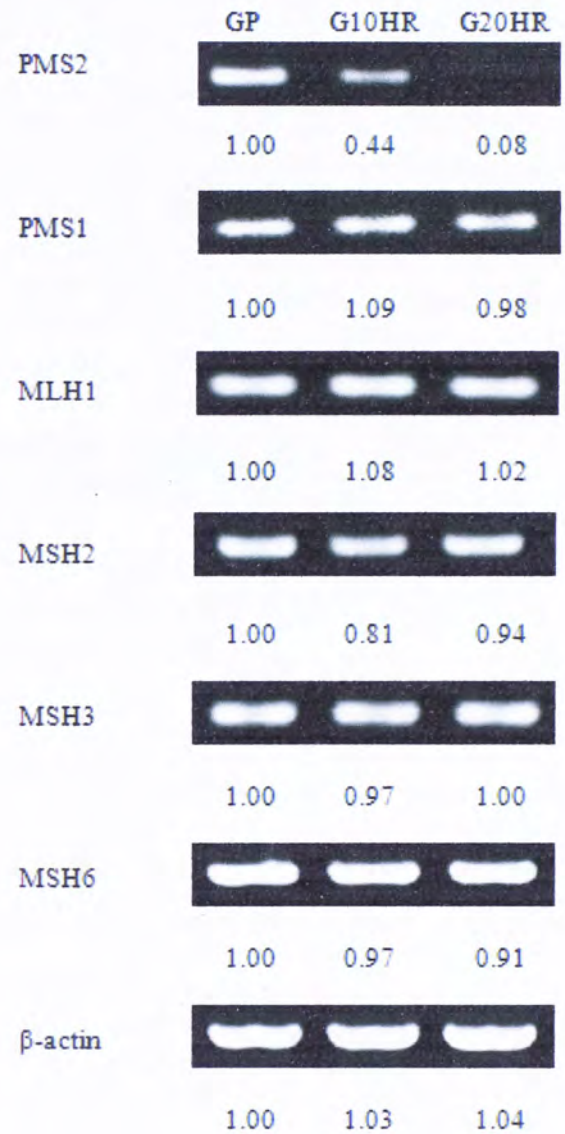


Fig. 4.3.2. The basal mRNA expression levels of DNA mismatch repair (MMR) genes in HepG2 cells. The expression of individual genes were measured by RT-PCR. The band intensity was analyzed with Image J 1.40g.

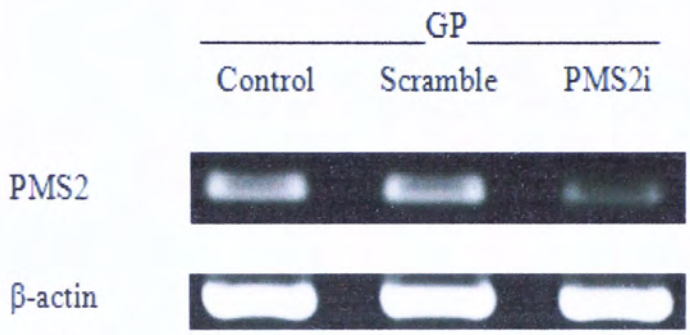


Fig. 4.3.3. The mRNA level of PMS2 after PMS2i transfection in GP cells.

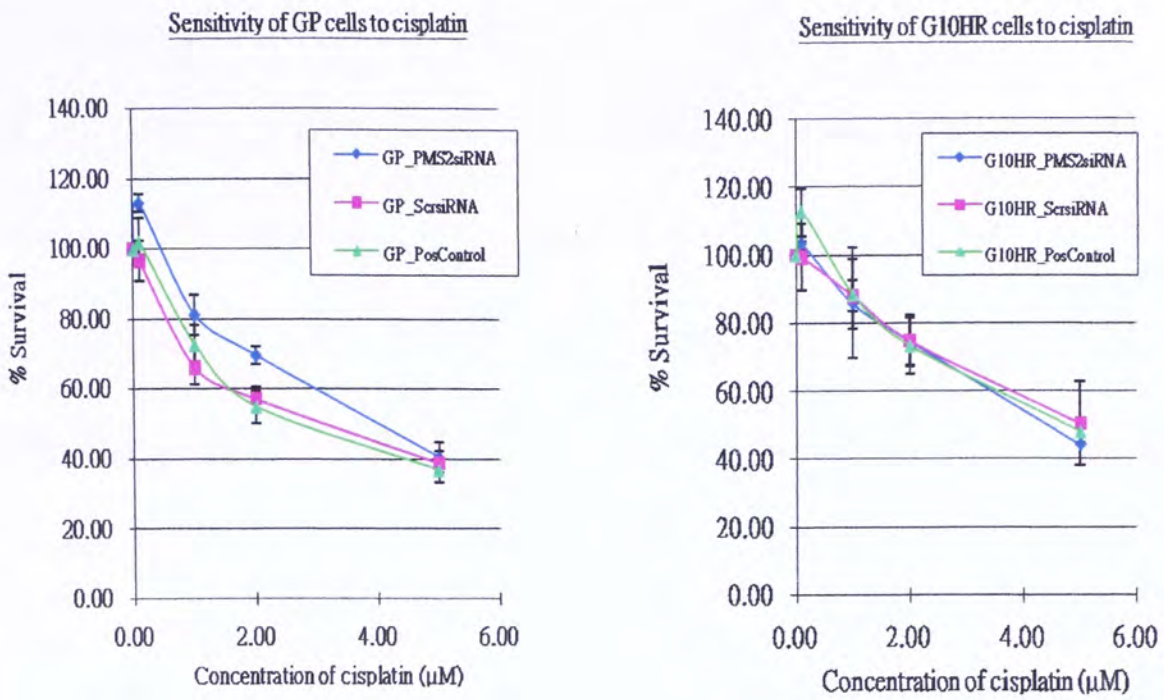


Fig. 4.3.4. The sensitivity of HepG2 cells to cisplatin after PMS2i transfection as measured by MTT assay. - ◆ - : cells that have transfected by PMS2 siRNA ; - ■ - : cells that have transfected by scramble siRNA; -- ▲ -- : cells without any transfection. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

4.3.4 Expression profile of MMR genes in the presence of 5-Aza-dC

DNMT inhibitor 5-aza-2'-deoxycytidine (5-Aza-dC) was then utilized to examine the effect of DNA methylation, one of epigenetic events, in the regulation of PMS2. It was stated that DNMT inhibitor can block the activity of DNMT resulting in hypomethylation of the gene's promoter and re-expression of that gene. After the addition of 5-Aza-dC to the HepG2 cells, the PMS2 expression level of both GP and G10HR cells was induced (Fig. 4.3.5). For the other 5 MMR genes, their mRNA expression levels seemed to remain more or less the same.

4.3.5 Drug sensitivity of HepG2 cells to cisplatin after the addition of 5-Aza-dC

The epigenetic up-regulation of PMS2 and the relationship between cisplatin resistance and PMS2 expression level was further confirmed by MTT assay. HepG2 cells were co-treated with 5-Aza-dC and cisplatin for 3 days and the results were shown in Fig. 4.3.6. All the three HepG2 cell lines became more resistant to cisplatin in the presence of 5-Aza-dC. The increase in the % survival was about 10% to 20%. The results suggested that the mRNA level of PMS2 seemed to directly correlate with cisplatin resistance in HepG2 cells. That means, the lower the mRNA level of PMS2, the greater the cisplatin resistance in the cells. In addition, hypopxia/reoxygenation treatment may down-regulate the expression of PMS2 by DNA methylation.

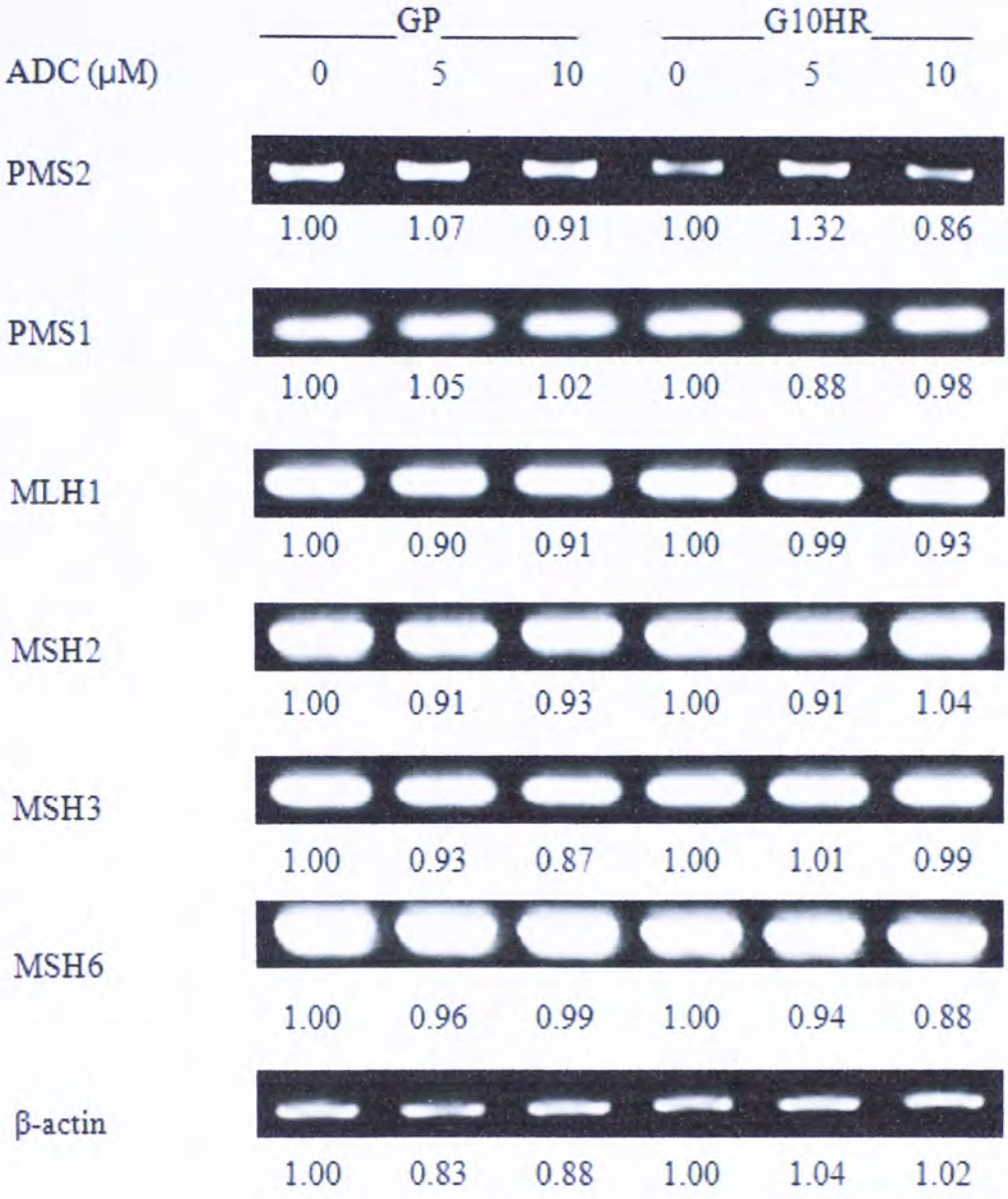


Fig. 4.3.5. The mRNA expression levels of MMR genes in HepG2 cells with or without the presence of 5-Aza-dC. The expressions of individual genes were measured by RT-PCR. The band intensity was analyzed with Image J 1.40g and the numbers showed the relative expression of the gene compared to the control (GP or G10HR), which is designated as 1.00.

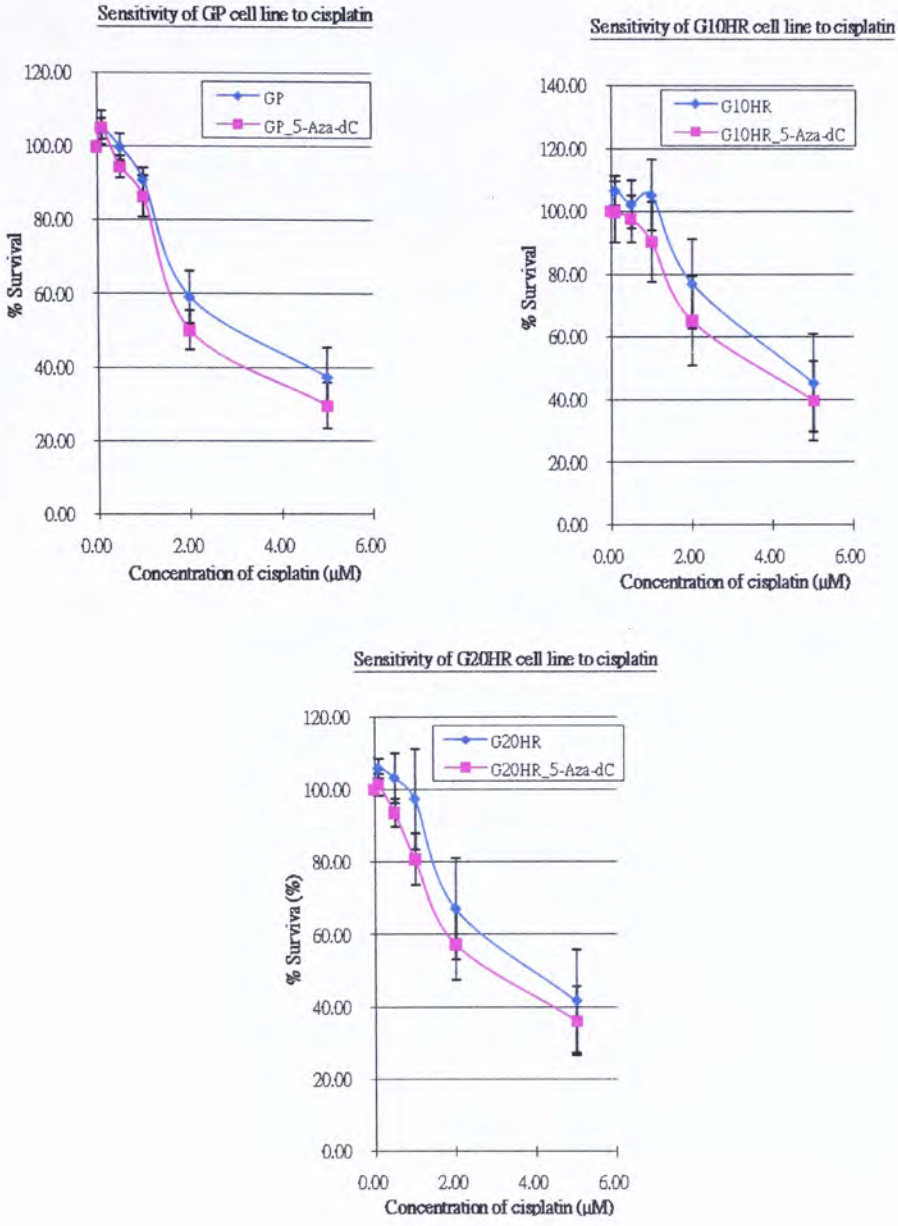


Fig. 4.3.6. The sensitivity of HepG2 cells to cisplatin in the presence of 5-Aza-dC. - ◆ - : cells treated with cisplatin only ; - ■ - : cells co-treated with cisplatin and 5-Aza-dC. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

4.3.6 Expression profile of MMR genes in the presence of trichostatin A

Another type of epigenetic event was histone acetylation. It is well known that the activity of HDAC can be inhibited by HDAC inhibitors like trichostatin A and hyperacetylation of histone proteins re-express the target gene. After the administration of trichostatin A, the expression level of PMS2 was found to be up-regulated in GP and G10HR cells respectively (Fig. 4.3.7). For the other 5 MMR genes, their mRNA expression levels seemed to remain more or less the same.

4.3.7 Sensitivity of HepG2 cells to cisplatin after the addition of trichostatin A

Similarly, the epigenetic regulation of cisplatin rsistance was further confirmed by MTT assay. HepG2 cells co-treated with trichostatin A and cisplatin was compared with HepG2 cells treated with trichostatin A only. Obviously, all the three HepG2 cells became more resistant to cisplatin in the presence of trichostatin A (Fig. 4.3.8). Thus, hypopxia/reoxygenation treatment may also down-regulate the expression of PMS2 by DNA acetylation that confer cisplatin resistance in HepG2 cells.

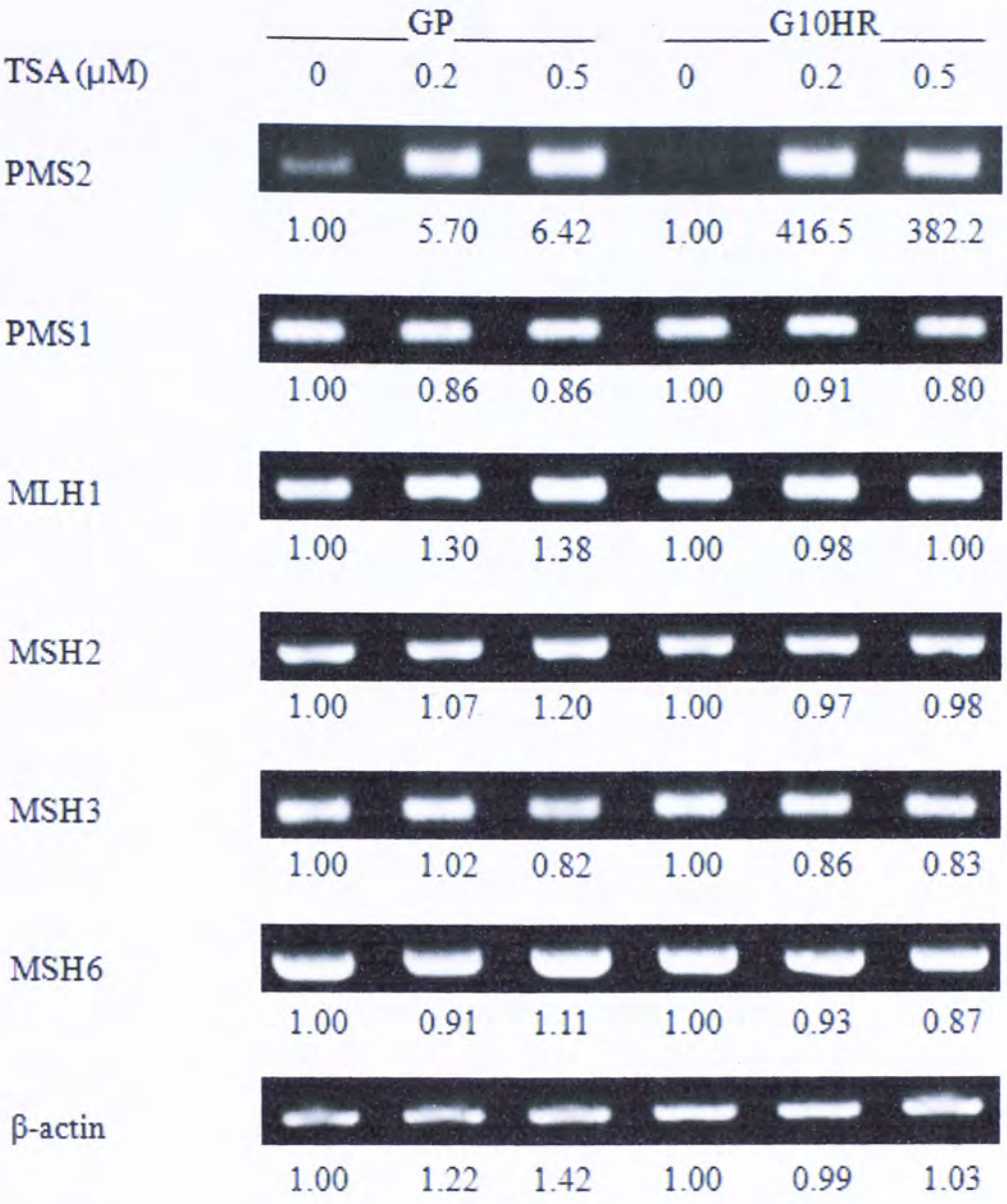


Fig. 4.3.7. The mRNA expression levels of MMR genes in HepG2 cells with or without the presence of TSA. The experiment of individual genes was measured by RT-PCR. The band intensity was analyzed with Image J 1.40g and the numbers showed the relative expression of the gene compared to the control (GP or G10HR), which is designated as 1.00.

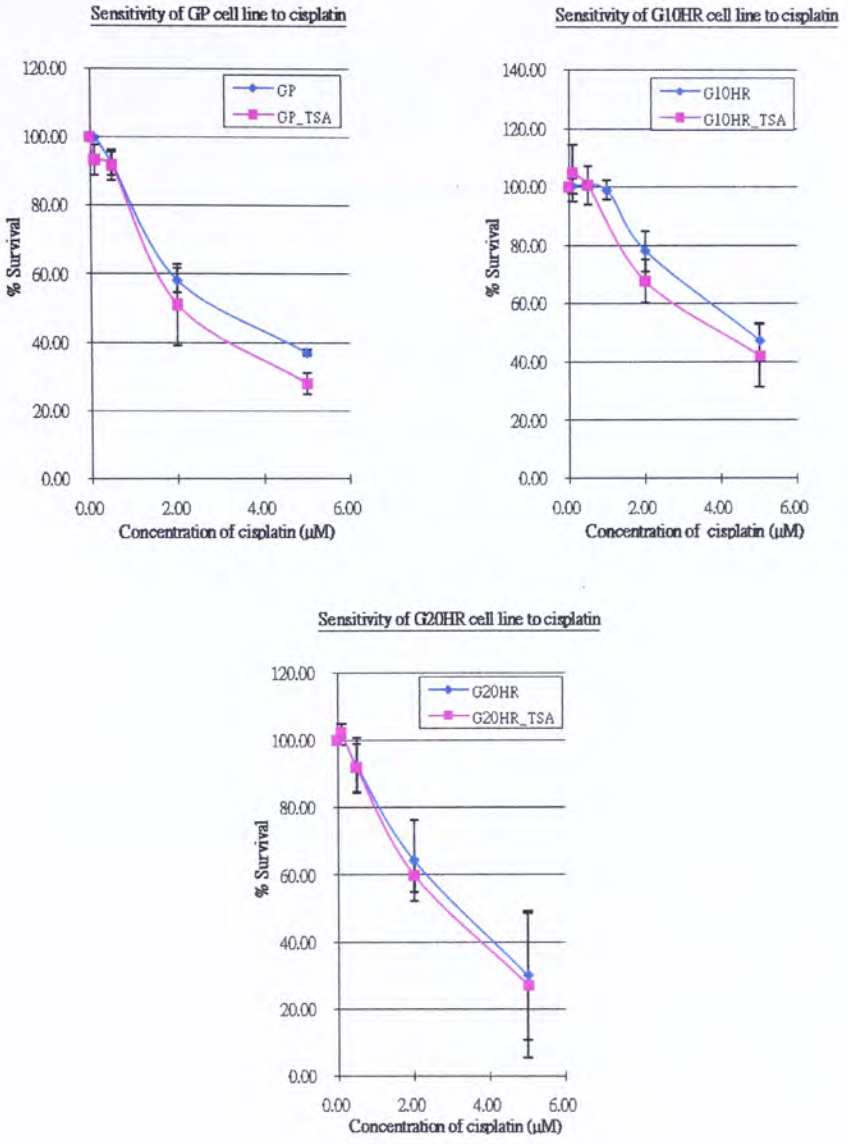


Fig. 4.3.8. The sensitivity of HepG2 cells to cisplatin in the presence of trichostatin A (TSA). - ◆ - : cells treated with cisplatin only; - ■ - : cells co-treated with cisplatin and TSA. Viability was expressed as a percentage of an untreated control. Error bars showed the standard deviation of at least 3 experiments.

4.4 Discussion

Although cisplatin has been discovered and utilized clinically for more than 40 years, there are still few other platinum-based compounds can replace it. Carboplatin and oxaliplatin are the only examples commonly used. However, there was an urge to find a new substitute for or modify cisplatin because of the increasing incidences of cisplatin resistance in cancer treatment. In addition, the side effects of cisplatin were severe including neurotoxicity [Verschraegen *et.al.*, 1995], vascular toxicity [Icli *et.al.*, 1993] and nephrotoxicity [Arany and Safirstein, 2003]. Thus, continuous effort is required to identify the mechanism of cisplatin resistance so as to find a better way of using platinum-based drugs.

In the present study, how hypoxia/reoxygenation controlled the epigenetic regulation of cisplatin resistance was determined. From the results of MTT assay (Fig. 4.3.1), G10HR and G20HR cells were found to be more resistant to cisplatin than that of GP cells. The resistance to cisplatin may be contributed by the hypoxia/reoxygenation treatment. Probably, hypoxia/reoxygenation affected the expressions of cisplatin-associated genes. Among a vast amount of genes, DNA repair genes have been suggested for some time to be important in directing cisplatin resistance. Dabholkar claimed that enhanced level of excision repair cross-complementation group 1 (ERCC1) may play a role in clinical resistance to

cisplatin [Dabholkar *et.al.*, 1992]. Besides, Kishi reported that reduced MLH1 expression was related to poor prognosis in esophageal cancers [Kishi *et.al.*, 2003]. In our HepG2 cell lines, the basal mRNA expression level of PMS2 was progressively down-regulated in G10HR and G20HR cells while the mRNA expression levels of the other five MMR genes were similar among all the three HepG2 cell lines (Fig. 4.3.2). After the knock down of PMS2 in GP cells, which had the highest basal level of PMS2 among the three HepG2 cell lines, GP cells became more resistant to cisplatin (Fig. 4.3.4). It seemed that the expression level of PMS2 correlated with the efficacy of cisplatin. In fact, the resistance to alkylating agents such as cisplatin was claimed to be associated with the reduction of PMS2 in human cancers such as breast cancer, melanoma [Francia *et.al.*, 2005], ovarian cancer [Drummond *et.al.*, 1996] and osteosarcoma [Perego *et.al.*, 1999]. However, a report showed that there was no association between MMR inactivation and cisplatin resistance in ovarian cancer cell lines [Helleman *et.al.*, 2006]. Thus, further investigation of their relationship was needed.

It was known that the expression patterns of numerous amounts of genes were changed under hypoxia. For example, the expression of pro-apoptotic gene Bnip3 was induced in the developing cortex by hypoxia [Sandau and Handa, 2007], the repression of MLH1 and MSH2 by hypoxia through shifting the activation of

c-Myc/Max to repression of Mad1/Max [Bindra and Glazer, 2007], etc. Other than gene mutation, epigenetic regulation was one of the possible mechanisms to alter the expression of a gene. Typically, epigenetic regulation of a gene can be brought by DNA methylation and histone deacetylation. For DNA methylation, hypermethylation of the promoter of a gene seemed to silence the expression of the gene. For instance, glutathione S-transferase P1 [Zhang *et.al.*, 2005], O6-methylguanine-DNA methyltransferase (MGMT) [Nakagawachi *et.al.*, 2003] and E-cadherin [Garinis *et.al.*, 2002] had been reported to be silenced by promoter hypermethylation. For histone deacetylation, hypoacetylation of histone proteins also seemed to silence the expression of a gene. For example, candidate tumor-suppressor gene DLEC1 was downregulated by histone hypoacetylation in human epithelial ovarian cancer [Kwong *et.al.*, 2006]. Likewise, the mRNA expression levels of PMS2 in GP and G10HR cell lines were re-expressed or up-regulated in the presence of DNMT inhibitor, 5-aza-2'-deoxycytidine (Fig. 4.3.5), or HDAC inhibitor, trichostatin A (Fig. 4.3.7). Furthermore, all the three HepG2 cell lines became more resistant to cisplatin after the addition of either one of the above inhibitors (Fig. 4.3.6 and 4.3.8).

In conclusion, our results suggested that hypoxia/reoxygenation may direct the epigenetic down-regulation of PMS2 by DNA methylation and histone deacetylation, resulting in cisplatin resistance. Besides, the expression of PMS2 was inversely

correlated with the efficacy of cisplatin treatment. The hypothesis can be further proved by methylation-specific PCR and Western blot analysis of histone proteins.

Chapter 5

The Role of PMS2 in Cisplatin-induced Apoptosis

5.1 Introduction

Undoubtedly, cisplatin works by forming DNA adducts. However, the exact events of how cisplatin-induced DNA damage and thus translated into durg-mediated cellular effects were unclear. As mentioned by Siddik, cisplatin-DNA adducts activate several signal transduction pathways consisting of cell cycle arrest, DNA synthesis inhibition and apoptosis. Among the potential pathways, apoptosis is of the most interest. Up till now, cisplatin-induced apoptosis was only known to be initiated or facilitated by more than 20 candidate proteins prior to DNA adducts formation but the extent of each of these proteins contributed to apoptosis was uncertain.

5.1.1 Apoptosis

Apoptosis was first introduced by John Kerr in 1972 and described as the formation of apoptotic bodies from a cell. Precisely speaking, apoptosis is a process of controlled, regulated and programmed cell death and it is different from necrosis, which is an uncontrolled cell death leading to lysis of cells. Morphologically, distinct changes of cytoplasm, nucleus and plasma membrane were observed during the early stage of apoptosis. In the cytoplasm, the endoplasmic reticulum dilates and the cisternae swell to form vesicles and vacuoles. In the nucleus, chromatin condenses, aggregates into dense compact masses and is fragmented to form apoptotic bodies. In the plasma membrane, cell junctions are disintegrated and blebbing occurs. Eventually, the cell rounds up and shrinks [Lawen, 2003].

Apoptosis is responsible for removal of damaged cell beyond repair, maintenance of homeostasis inside our body, tissue development and regulation of immune cells, etc. All these events are mediated by two extensively studied apoptotic pathways: the extrinsic or receptor-mediated pathway, and, the intrinsic or mitochondrial pathway.

5.1.2 Extrinsic pathway of apoptosis

Extrinsic pathway is triggered by binding of extracellular ligands to death receptors located on the plasma membrane of the cell. There are several kinds of death receptors: Fas, tumor-necrosis factor receptor (TNF-R) 1, death receptor (DR) 4 and 5, CAR1 and p75. In Fas signaling pathway, the Fas ligand (FasL) binds to Fas upon T-cell activation and resulting in receptor trimerization. This signals the binding of Fas-associated death domain protein (FADD) to Fas. Together with pro-caspase 8, a complex called death inducing signaling complex (DISC) is formed. DISC can transactivate pro-caspase 8 to active caspase 8 which then cleaves the executioner caspases such as caspase 3 and apoptosis starts (Fig. 5.1.1).

5.1.3 Intrinsic pathway of apoptosis

Intrinsic pathway is a mitochondria-dependent process that is activated by stress such as oxidative stress or cytotoxic drugs. Upon activation, BH3-only proteins such as BAD and BIM trigger the oligomerization of BAX and BAK. The mitochondrial potential is then lost and cytochrome c is released. In addition, second mitochondrial-derived activator of caspase (SMAC)/Diablo proteins are released into

the cytosol which bind to and deactivate inhibitor of apoptosis proteins (IAPs) so that apoptosis can proceed. The released cytochrome c binds to the Apoptotic Protease Activating Factor-1 (Apaf-1) and leads to the formation of apoptosome. The apoptosome then activates the pro-caspase 9 followed by other effector caspases. Eventually, the activated effector caspases cleave the cellular substrates to carry out apoptosis (Fig. 5.1.1).

5.1.4 Cisplatin-induced apoptosis

The cisplatin-induced apoptosis seemed to involve p53. As reported by Jayaraman, HMG proteins facilitated the binding of p53 to DNA upon recognition of DNA damage [Jayaraman *et.al.*, 1998]. Afterwards, p53 transactivated several kinds of genes such as the pro-apoptotic gene, Bax. Bax was then translocated from the cytosol to the mitochondria. The loss of mitochondrial potential released proteins such as cytochrome c which activated the pro-caspase 9 and 3 substantially and apoptosis ensued [Makin *et.al.*, 2001]. This apoptotic pathway depended on the Bax:Bcl-2 ratio. This ratio was increased when the apoptosis occurred [Del Bello *et.al.*, 2001]. On the other hand, cisplatin-induced apoptosis also proceeded through Fas receptor. Binding of FasL to Fas receptor activated caspase 8 and eventually caspase 3, resulting in apoptosis (Fig. 5.1.2) [Micheau *et.al.*, 1997]. This apoptotic pathway is also p53-dependent [Muller *et.al.*, 1998]. Although both ciplatin-induced apoptotic pathways depended on p53, some reports suggested the malfunction of p53 sensitizes tumor cells to cisplatin but not makes them more resistant [Fan *et.al.*, 1995].

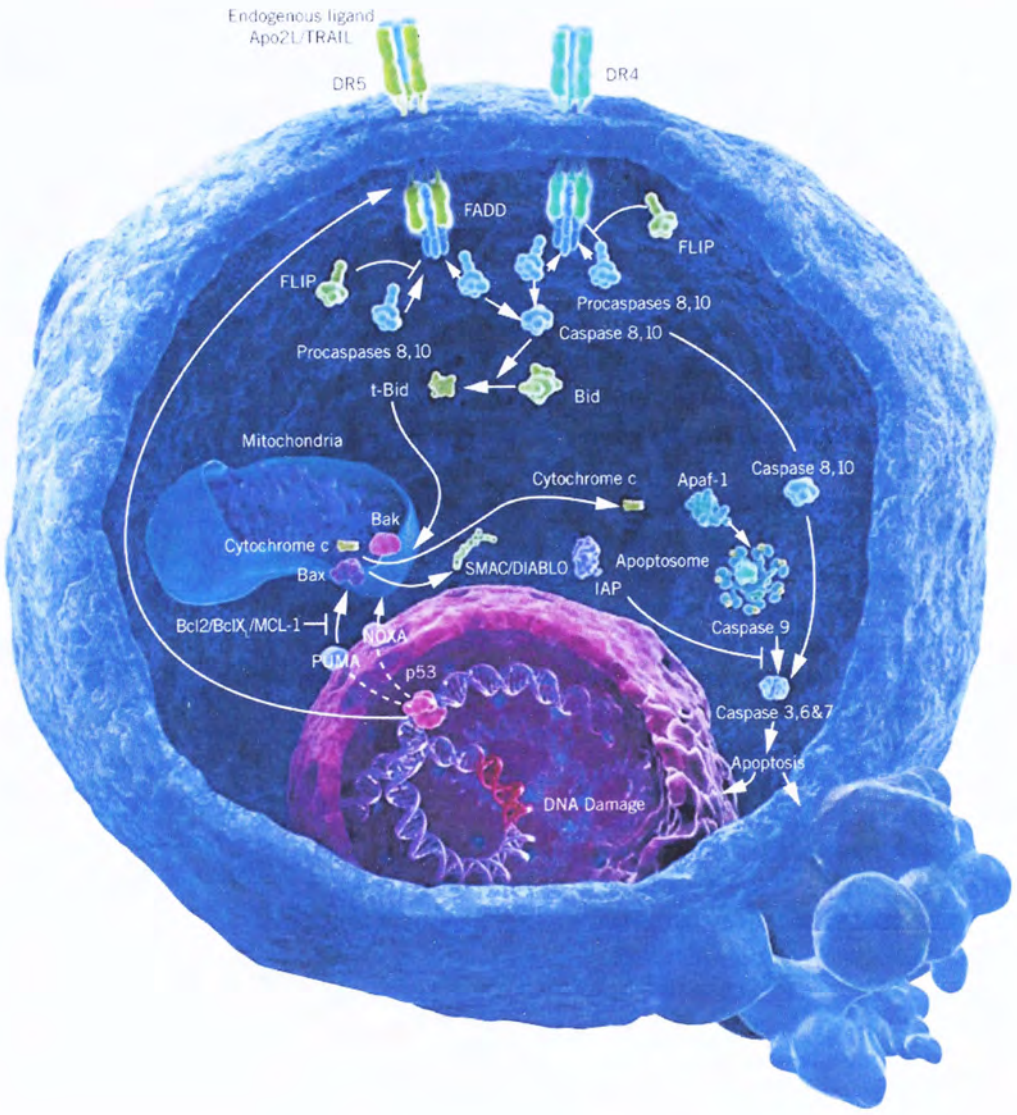


Fig. 5.1.1 A diagram showing the process of apoptosis. (Adopted from the website of "http://www.biooncology.com")

5.1.5 MMR and apoptosis

When the extent of DNA damage was beyond a critical threshold, apoptosis was triggered by MMR proteins. Nonetheless, there were still not much articles describing direct apoptotic signaling by MMR proteins. It has been reported that MSH2 protein interacted with ATR and regulated the phosphorylation of Chk1 [Wang and Qin, 2003].

The role of MMR proteins as a direct apoptotic signaling is still so loose. Besides, MMR proteins seem to be upstream signaling molecules participating in apoptosis. In the previous chapter, it was demonstrated that PMS2 played a critical role in the susceptibility of cisplatin treatment. We proposed that PMS2 may act as a direct signaling molecule in cisplatin-induced apoptosis. Moreover, the hypoxia/reoxygenation treatment may alter the expression of signaling molecules along the cisplatin-induced apoptosis.

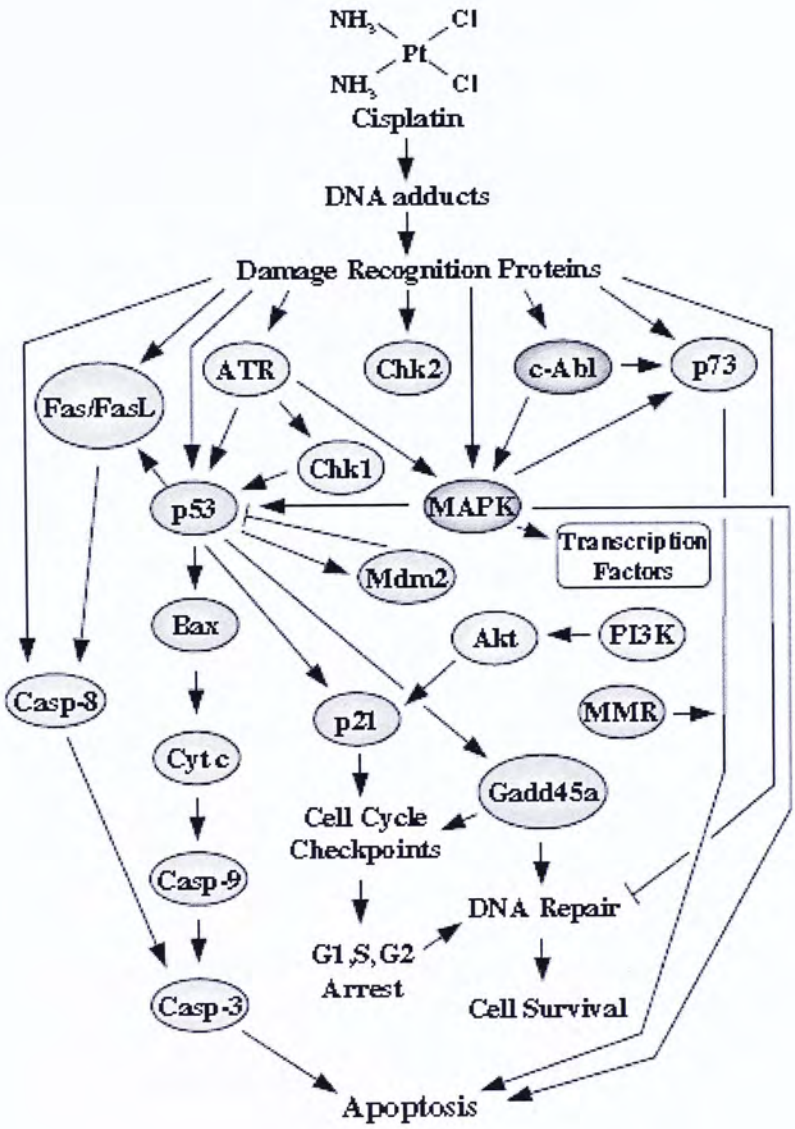


Fig. 5.1.2. The suggested pathways involved in cisplatin-induced apoptosis [Siddik, 2003].

5.2 Materials and Methods

5.2.1 Cell culture

Please refer to 4.2.1. All the assays performed beyond were in air.

5.2.2 Flow cytometric analysis of apoptosis

HepG2 cells were seeded with a density of $2x10^5$ on 60-mm dishes for 2 days and then treated with 2μM cisplatin with or without the presence of PMS siRNA for 3 days. Cells were then washed and collected in PBS and stained with 5μl Annexin-V-GFP fusion protein and 1mg/ml PI in binding buffer (10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$) for 15 minutes at room temperature in dark. Apoptotic cells were stained by Annexin-V-GFP fusion protein with green fluorescence emission while the nuclei of the apoptotic cells were stained by PI with red fluorescence emission. The fluorescence signal of the cells was analyzed by FACSort cytometer and 10^5 cells were read for each sample. Finally, the graphs were produced by software WinMDI 2.8 with the x parameter being FITC-A (Annexin-V-GFP fusion protein) and the y parameter being PerCP-Cy5-5-A (PI).

5.2.3 Oligonucleotide transfection

Please refer to 4.2.4.

5.2.4 Western blot analysis

Please refer to 3.2.4.

5.2.5 Drug and antibodies

Cisplatin was purchased from Sigma. Antibodies targeting ß-actin, Bcl-2, Bcl-xL, Bax, caspase 3, cytochrome c, FADD and Fas were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies targeting BID, caspase 8 and caspase 9 were purchased from Stressgen (San Diego,CA). Antibody targeting p53 was purchased from Oncogene. Caspase inhibitors 3, 8 and 9 were purchased from Merck.

5.3 Results

5.3.1 Cisplatin induced apoptosis

In chapter 4, it has been shown that G10HR cells were more resistant to cisplatin than GP cells. It was known that cisplatin exerted its cytotoxicity by causing DNA damage and eventually leading to apoptosis. Thus, it was expected that the sensitivity of cisplatin may directly correlated with the extent of apoptosis. From the results of flow cytometry, fewer G10HR cells were found to undergo apoptosis compared with that of GP cells (Fig. 5.3.1).

5.3.2 Knockdown of PMS2 by siRNA

As demonstrated in Chapter 4, PMS2 played a critical role in mediating cisplatin cytotoxicity. It was speculated that PMS2 may act as a direct signaling molecule in the cisplatin-induced apoptosis. Interestingly, fewer GP and G10HR cells underwent apoptosis after the expression of PMS2 was knocked down by PMS2i transfection (Fig. 5.3.1). In order to further confirm the role of PMS2 in cisplatin-induced apoptosis, the basal expressions of both anti-apoptotic and pro-apoptotic molecules were investigated.

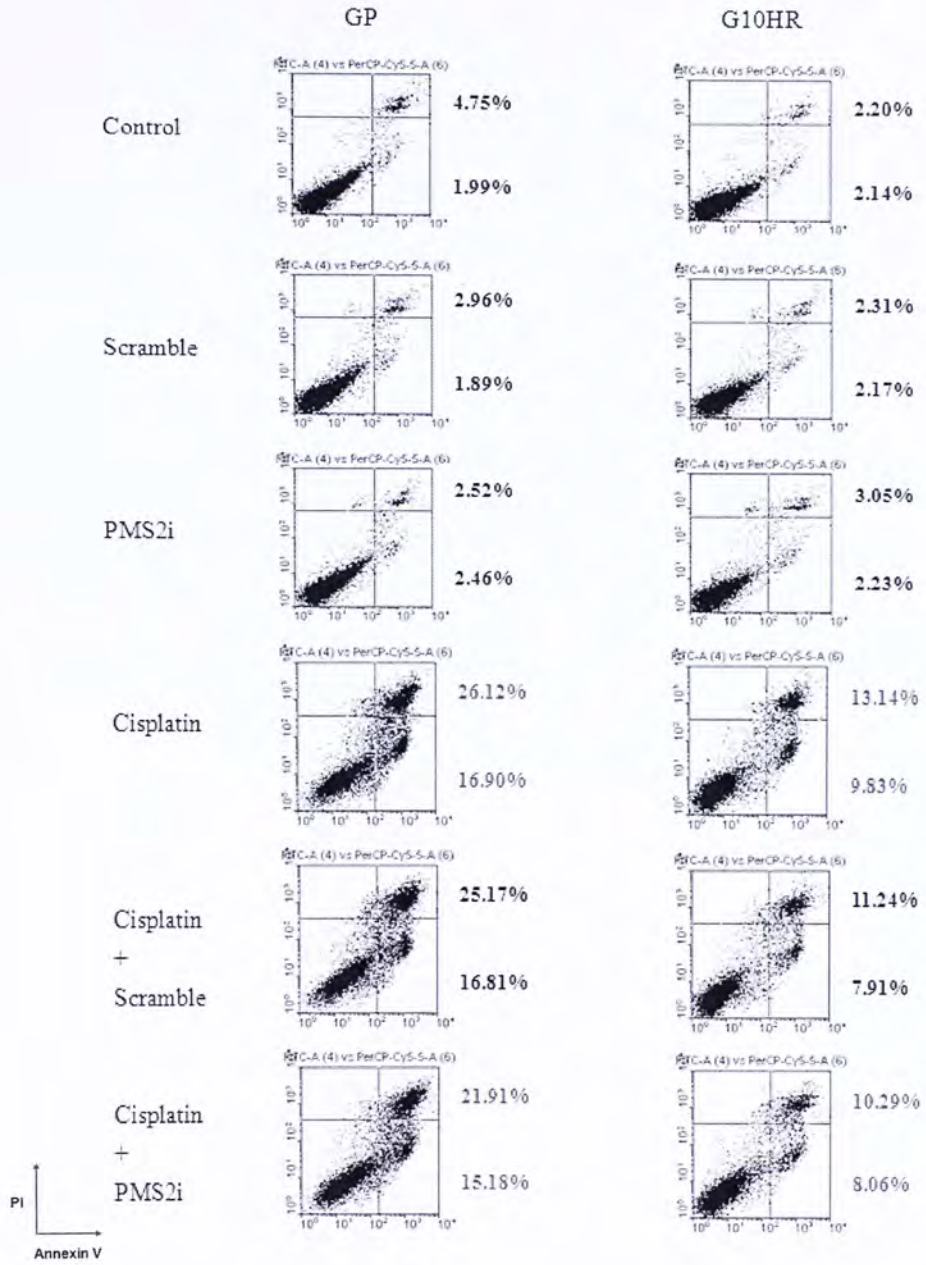


Fig. 5.3.1. Apoptosis of HepG2 cells with the treatment of cisplatin after the knockdown of PMS2. The graphs were divided into four quarters: viable cells were defined as annexin-V and PI double-negative events; early apoptotic cells were defined as annexin-V positive event; late apoptotic cells were defined as annexin-V and PI double-positive events.

5.3.3 Cisplatin-induced apoptosis involved caspases

Before looking into the expression levels of apoptotic proteins, it was necessary to see whether caspases were involved in cisplatin-induced apoptosis. The inhibitors of the three common caspases, caspases -3, -8 or -9, were incubated together with cisplatin in the HepG2 cells and flow cytometry was performed. The result showed that fewer GP or G10HR cells underwent apoptosis after the addition of either one of the above caspase inhibitor upon cisplatin treatment (Fig. 5.3.2, 5.3.3 and 5.3.4). In short, cisplatin-induced apoptosis involved caspases -3, -8 and -9.

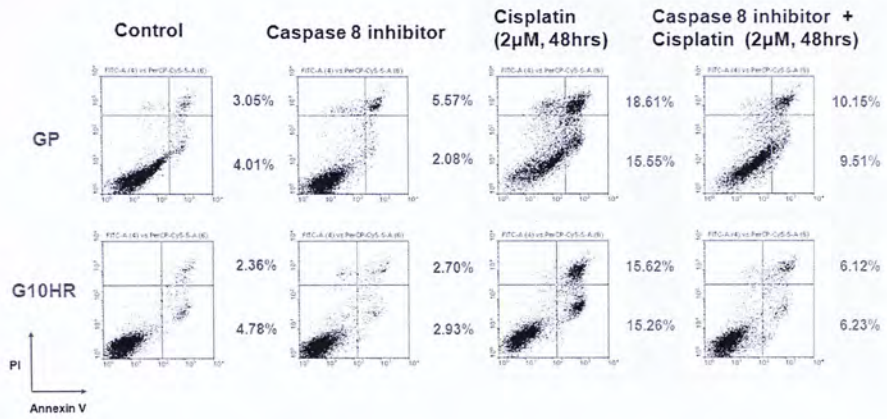


Fig. 5.3.2. The cisplatin treatment in GP and G10HR cells in the absence or presence of 30μM caspase 8 inhibitor.

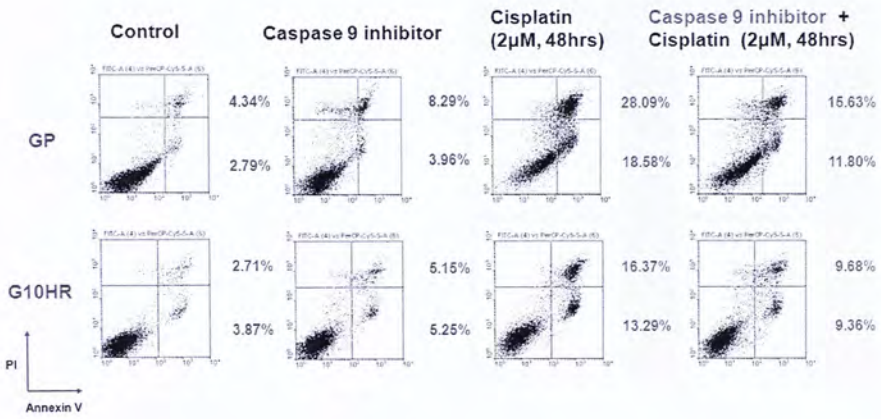


Fig. 5.3.3. The cisplatin treatment in GP and G10HR cells in the absence or presence of 30μM caspase 9 inhibitor.

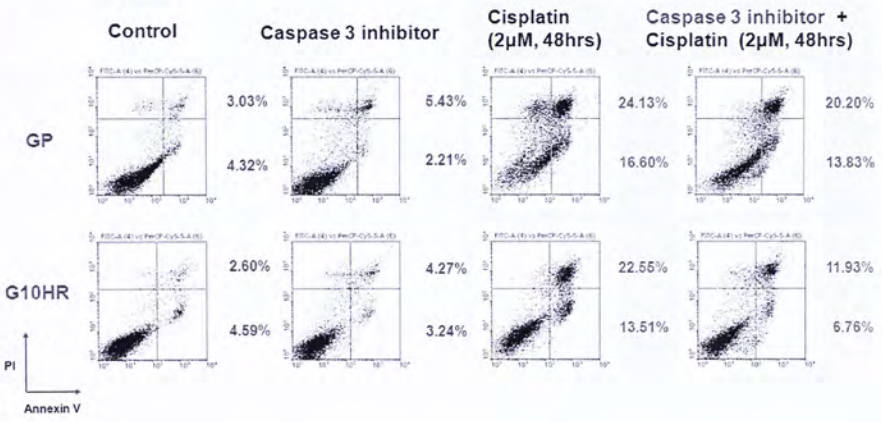


Fig. 5.3.4. The cisplatin treatment in GP and G10HR cells in the absence or presence of 30μM caspase 3 inhibitor.

5.3.4 Protein expressions of anti-apoptotic genes

In order to elucidate how G10HR cells were adapted to cisplatin-induced apoptosis, both the expressions of anti-apoptotic and pro-apoptotic proteins were examined. For the anti-apoptotic proteins, they comprised Bcl-2 and Bcl-xL. By Western blot analysis, the basal expression of Bcl-2 in G10HR cells was significantly higher than that of GP cells but the basal expression of Bcl-xL was more or less the same in GP and G10HR cells (Fig. 5.3.5).

5.3.5 Protein expressions of pro-apoptotic genes

For pro-apoptotic proteins, they included p53, Fas, FADD, Bax, Bid, cytochrome c, caspase 3, caspase 8 and caspase 9. Fas, FADD and caspase 8 were mainly responsible for the extrinsic pathway of apoptosis while Bax, Bid, cytochrome c and caspase 9 were mainly responsible for the intrinsic pathway of apoptosis. Among them, down-regulation of Fas, FADD, caspase 3 and caspase 9 was observed in G10HR cells compared with that of GP cells. For Bax, Bid and cytochrome c, both GP and G10HR cells had similar basal expression levels (Fig.5.3.6). Unexpectedly, the basal expression levels of p53 and caspase 8 were higher in G10HR cells than that of GP cells. Taken along with the data showing fewer G10HR cells underwent cisplatin-induced apoptosis than that of GP cells, it seemed that cisplatin-induced apoptosis proceeded through the extrinsic pathway.

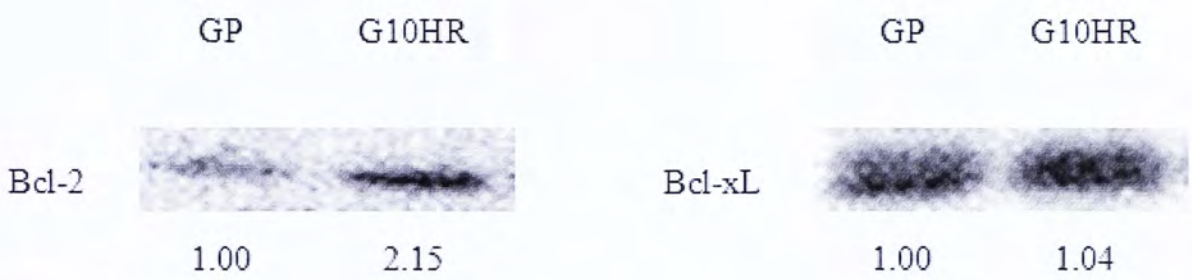


Fig. 5.3.5. The basal protein expression levels of anti-apoptotic proteins in HepG2 cells. The band intensity was analyzed with Image J 1.40g and the values indicated the relative expression of the proteins compared to the control (GP), which was designated as 1.00.

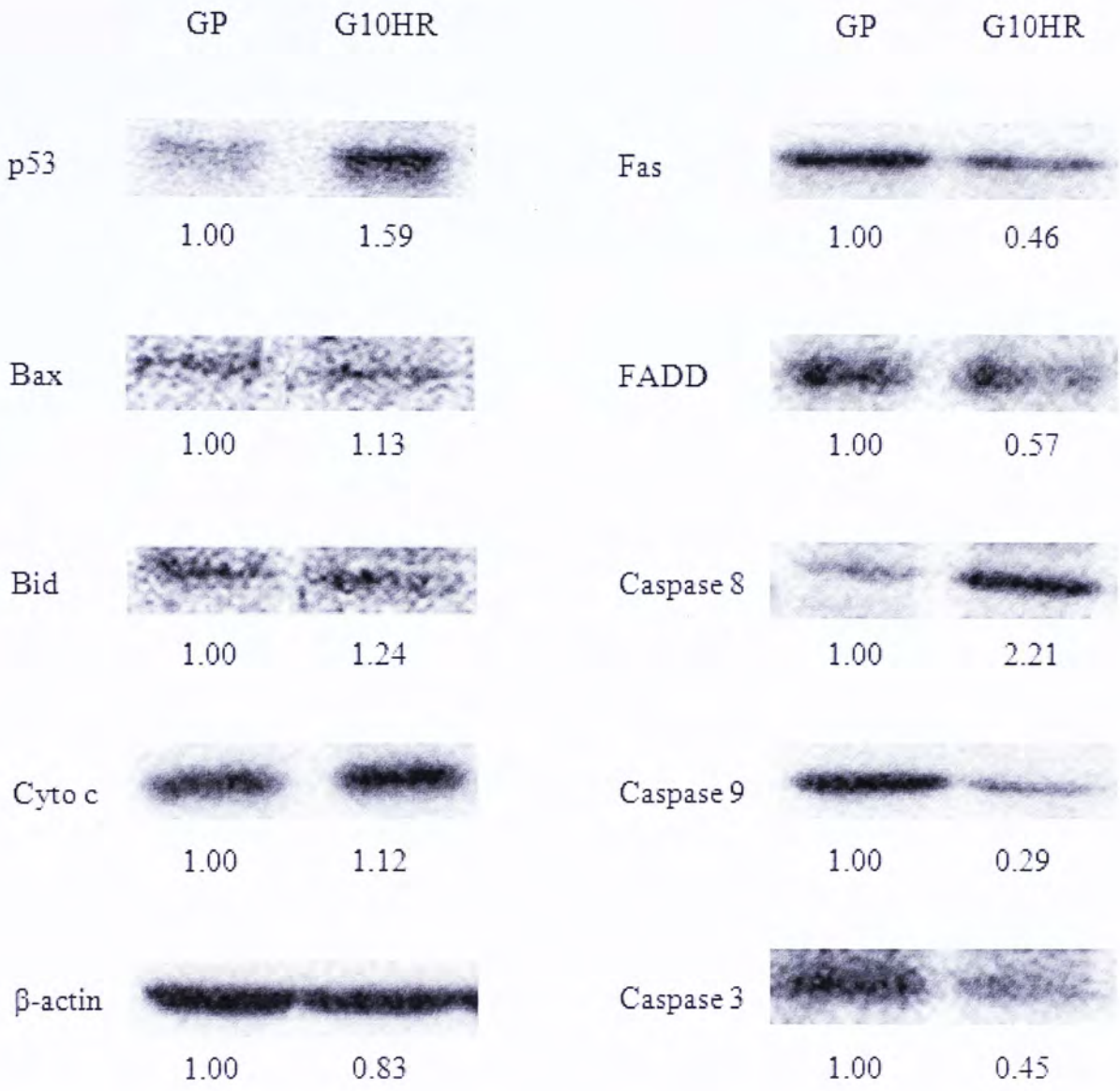


Fig. 5.3.6. The basal protein expression levels of pro-apoptotic proteins in HepG2 cells. The band intensity was analyzed with Image J 1.40g and the values indicated the relative expression of the proteins compared to the control (GP), which was designated as 1.00.

5.3.6 Protein expressions of apoptotic proteins after knockdown of PMS2

Up till now, no one had reported the role of PMS2 as a direct signaling molecule in cisplatin-induced apoptosis yet. Hence, PMS2 was knocked down by siRNA and see if its level affected the protein expression levels of apoptotic proteins. After the PMS2 level was knocked down, p53, Fas, FADD, Bax, Caspase 8 and Caspase 3 were down-regulate while only Bcl-2 was up-regulated. For Bid and caspase 9, the expression levels remained the same.

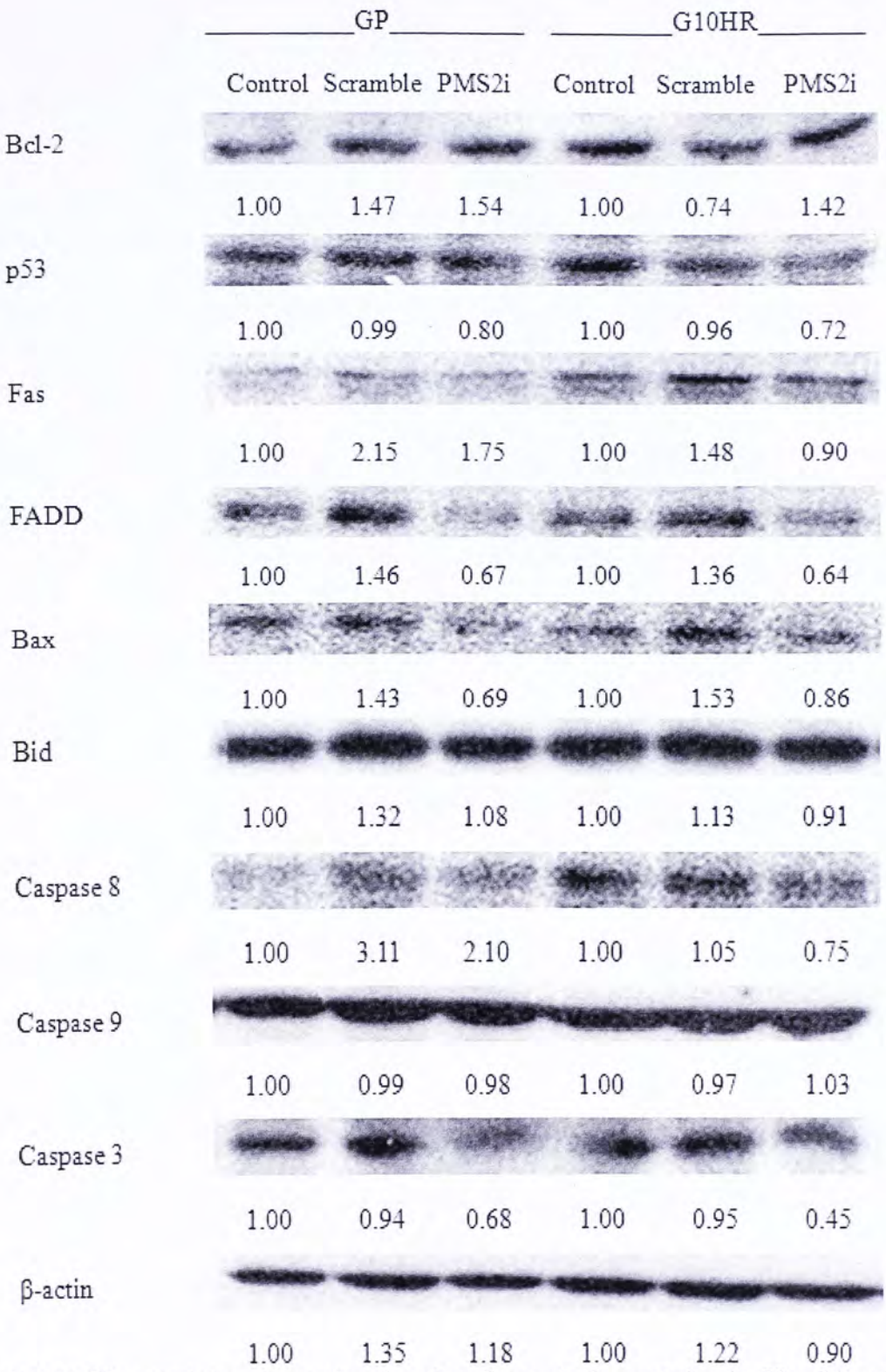


Fig. 5.3.7. The protein expression levels of apoptotic proteins in HepG2 cells after the knockdown of PMS2 by siRNA transfection. The band intensity was analyzed with Image J 1.40g and the numbers showed the relative expression of the proteins compared to the control (GP/G10HR), which was designated as 1.00. The difference in the expression could be related to Fig. 5.3.5 and 5.3.6.

5.4 Discussion

PMS2, which forms heterodimers with MLH1, is one of the mismatch repair genes responsible for post-replication correction of DNA mispairs. Extensive amount of reports were discussing the association of several diseases with the mutation of PMS2 which resulted in microsatellite instability. For instance, mutation in PMS2 was well known to cause hereditary nonpolyposis colorectal carcinoma (Lynch syndrome) [Hendriks *et.al.*, 2006], Turcot's syndrome [De Rosa *et.al.*, 2000], and trigger the formation of café-au-lait spots that leaded to childhood cancers [Kratz *et.al.*, 2008]. However, only a few reports were talking about the role of PMS2 as a direct signaling molecule to initiate apoptosis. Accordingly, PMS2 mediated the ionizing radiation-induced apoptosis via a p53-independent pathway [Zeng *et.al.*, 2000], stabilized p73 that enhanced DNA damage-induced apoptosis [Shimodaira *et.al.*, 2003] and had p53-response elements that activated p53 upon severe DNA damage [Chen and Sadowski, 2005].

In the previous chapter, it has been demonstrated that the sensitivity of cisplatin was directly correlated with the expression of PMS2. At the moment, it further showed that the sensitivity of cisplatin was directly correlated with the extent of apoptosis in which fewer G10HR cells were found to undergo apoptosis compared with that of GP cells (Fig. 5.3.1). In addition, knockdown of PMS2 in HepG2 cells led to the downregulation of ciplatin-induced apoptosis (Fig. 5.3.2). To summarize the above data, it was hypothesized that PMS2 may directly signal particular apoptotic protein(s) to initiate cisplatin-induced apoptosis. It was proven that cisplatin-induced apoptosis may depend on the activity of caspases such as caspases -3, -8 and -9 (Fig. 5.3.2, 5.3.3 and 5.3.4).

In order to elucidate how PMS2 signaled the apoptotic protein and how hypoxia/reoxygenation treatment affected the cisplatin-induced apoptosis, the basal expression levels of apoptotic proteins and their corresponding expression levels after the knockdown of PMS2 were examined. Apoptotic proteins can be divided into two distinct groups: anti-apoptotic and pro-apoptotic proteins. Pro-apoptotic proteins are proteins that are responsible for initiating apoptosis by three types of stimuli: the deprivation of survival factors, signals generated upon ligand-receptor binding, or cell damaging stress [Jarpe *et.al.*, 1998]. Examples of pro-apoptotic proteins include p53, Fas, FADD, Bax, Bid, cytochrome c, caspase 3, caspase 8, caspase 9. In contrast, anti-apoptotic proteins are proteins that protect cells from undergoing apoptosis by blockage of caspase activity, inhibition of the pro-apoptotic proteins signaling, or removal of the apoptotic stimuli. Examples of anti-apoptotic proteins include Bcl-2 and Bcl-xL.

From the results, the basal expression levels of pro-apoptotic proteins, Fas, FADD, caspase 3 and caspase 9 in G10HR cells were significantly lower than that in GP cells (Fig. 5.3.5). On the other hand, the basal expression levels of anti-apoptotic protein Bcl-2 in G10HR cells was much higher than that in GP cells and that of Bcl-xL was similar in both cell lines (Fig.5.3.6). Unexpectedly, the basal expression level of pro-apoptotic proteins, p53 and caspase 8, were much higher in G10HR cells than that of GP cells which was contradicted to the results of the sensitivity of cislpatin treatment (Fig. 5.3.5). Generally, high basal expression levels of pro-apoptotic proteins together with low basal expression levels of anti-apoptotic proteins result in greater extent of apoptosis upon cytotoxic drug treatment. Therefore, it was suspected the p53 protein in G10HR cells may be mutated as mutant p53 protein

function as anti-apoptotic protein. In conclusion, hypoxia/reoxygenation treatment seemed to down-regulate the expression levels of pro-apoptotic proteins and up-regulate the expression levels of anti-apoptotic proteins which prevented G10HR cells from carrying out apoptosis and made them more resistant to cisplatin therapy.

After PMS2 was knocked down by siRNA transfection, the expression of Fas, FADD, Bax, caspase 8 and caspase 3 was reduced while the expression of Bcl-2 and was enhanced. For Bid and Caspase 9, their expression levels were more or less the same before and after siRNA transfection (Fig. 5.3.7). The results suggested that PMS2 may act as a direct signaling molecule in cisplatin-induced apoptosis. The proposed pathway of cisplatin-induced apoptosis was presented in Fig, 5.4.1. It was hypothesized that cisplatin mediated apoptosis through the extrinsic pathway by interacting with Fas, either by binding with Fas or transactivating the expression of Fas. This signaled the binding of FADD to Fas receptor and subsequently combined with pro-caspase 8 to form a complex called DISC. DISC then transactivated pro-caspase 8 to active caspase 8 which cleaved the executioner caspase, caspase 3, and apoptosis was initiated consequently. In the future, the promoter activity of Fas will be investigated by Luciferase Reporter Assay so as to further confirm the above hypothesis.

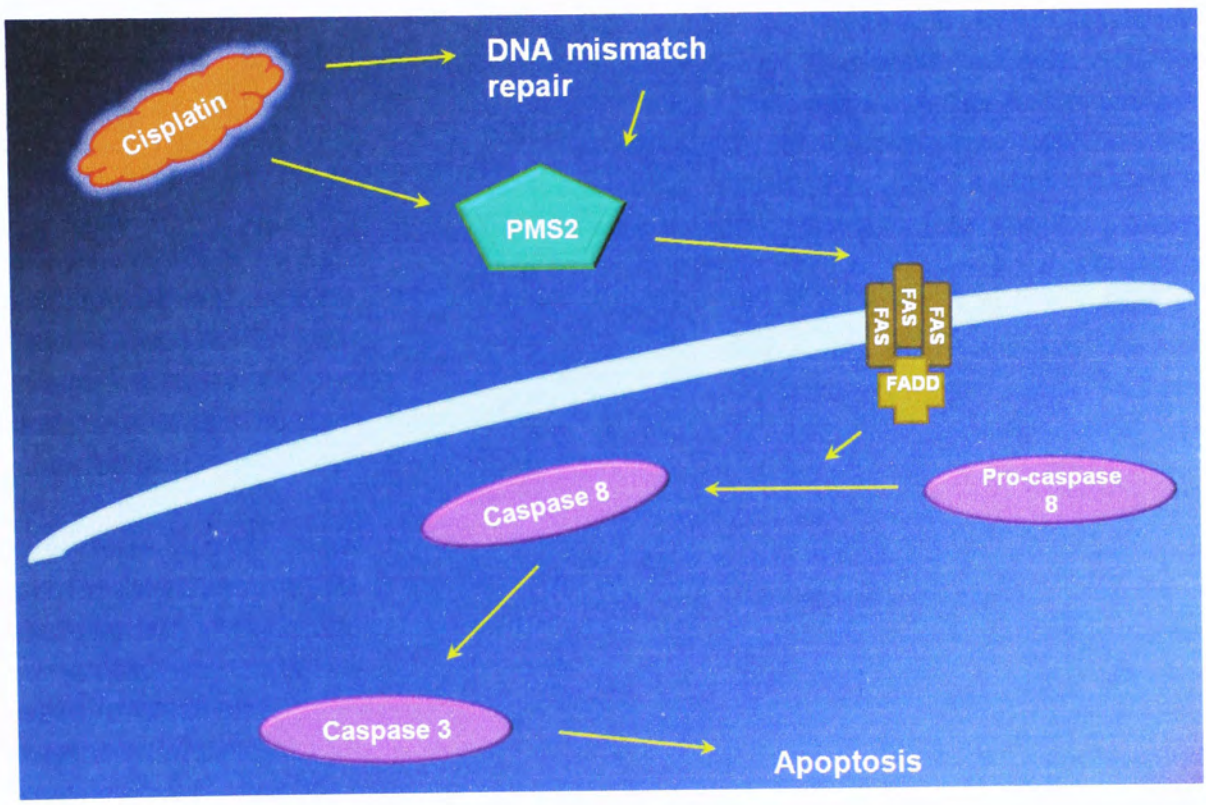


Fig. 5.4.1. The proposed pathway of cisplatin-induced apoptosis signaled by PMS2.

Chapter 6

General Discussion and Conclusion

In order to have a better treatment outcome for cancer, continuous work should be done to obtain a clear picture for the treatment resistance developed in cancer cells. It was found that the permanent change in the transcriptome was the main reason for the development of drug resistance in hypoxia/reoxygenation treated HepG2 and A431 carcinoma cells.

6.1 Diverse sensitivity for hypoxia/reoxygenation treated cells to anticancer drugs

The sensitivity to common clinically used cancer therapeutics was studied in HepG2 and A431 cells. It consisted of platinum drugs, topoisomerase I or II inhibitors, antibiotic, cytokines, antifolate drug, mitotic inhibitors and oxidizing agent. In general, both HepG2 and A431 cells became more resistant to chemotherapy after hypoxia/reoxygenation treatment. In details, G10HR and G20HR were more resistant to cisplatin, carboplatin, camptothecin, 10-hydroxy camptothecin and methotrexate while A10HR and A20HR were more resistant to carboplatin, doxorubicin, hydrogen peroxide, IFNα and IFNγ. The details for the resistance mechanisms of the above drugs were required to be further studied and that may hint for the direction of research discussed in Chapter 2.4. It was believed that the reasons for the resistance mechanisms were multifactorial. The therapeutic target was advised to be focused on the gene or pathway that had the greatest contribution for the resistance mechanism. In addition, it was observed that different cancer cell lines had diverse change in the sensitivity of drugs after hypoxia/reoxygenation treatment.

6.2 Resistance mechanism of doxorubicin in A10HR and A20HR cells

Among all the drugs examined, doxorubicin was one of the drugs that was chosen for an in-depth study. Although overexpression of mdr1 was the most frequently mentioned reason for the doxorubicin resistance in cancer cells, no report illustrated the resistance mechanism of doxorubicin in cancer cells with hypoxia/reoxygenated treatment yet. We found that the hypoxia/reoxygenation treated A10HR and A20HR cells were more resistant to doxorubicin than the normoxic AP cells and the resistance was related to the overexpression of mdr1 gene. It was believed that hypoxia/reoxygenation treatment changed the methylation status of the promoter region of mdr1 gene in A10HR and A20HR cells. The hypomethylated mdr1's promoter facilitated the transcription of mdr1 mRNA and subsequently more P-glycoprotein was translated. Then more doxorubicin molecules could be pumped out of the carcinoma cells and thus reduced the intracellular concentration of doxorubicin upon doxorubicin treatment. Thereby, more carcinoma cells survived and they became resistant to doxorubicin.

6.3 Resistance mechanism of cisplatin in G10HR and G20HR cells

Another drug picked up for the in-depth study of its resistance mechanism in hypoxia/reoxygenation treated cells was cisplatin. Despite its long history of usage in cancer patients, extensive amount of reports about cisplatin resistance urged to study its resistance mechanism so as to find out a better treatment scheme. In the present study, hypoxia/reoxygenation treated G10HR and G20HR cells were found to be more resistant to cisplatin than that of the normoxic GP cells. Further investigation

revealed that the cisplatin resistance was somehow related to the expression level of one particular mismatch repair gene, PMS2. Unlike the case in doxorubicin resistance, the expression level of PMS2 was significantly down-regulated after hypoxia/reoxygenation treatment. By the technique of RNAi interference, it was proven that reduced level of PMS2 conferred cisplatin resistance in HepG2 cells. Furthermore, hypoxia/reoxygenation was suspected to down-regulate the expression of PMS2 in HepG2 cells through epigenetic regulation such as DNA methylation and histone deacetylation.

6.4 The role of PMS2 as a direct signaling molecule and the alteration of apoptotic proteins in cisplatin-induced apoptosis

As a lot of drugs killed the cancer cells through particular events that eventually triggered apoptosis, it is therefore worth finding of any differences in the expressions of different apoptotic genes in hypoxia/reoxygenation treated cells which may alter the extent of apoptosis upon drug treatment. Beforehand, it was found that the sensitivity to cisplatin was directly correlated with the extent of apoptosis in which fewer G10HR cells were found to undergo apoptosis compared with that of GP cells. Besides, the cisplatin-induced apoptosis was mediated by PMS2. From the basal expression profile of GP and G10HR cells, it seemed that hypoxia/reoxygenation treatment rendered the HepG2 cells down-regulating the pro-apoptotic proteins such as Fas, FADD, caspase 9 and caspase 3 but up-regulating the anti-apoptotic proteins such as Bcl-2. That may explain the resistant property of G10HR cells towards drugs such as cisplatin. Moreover, PMS2 was suspected to act as a direct signaling molecule in mediating the cisplatin-induced apoptosis as the

knockdown of PMS2 altered the expression of apoptotic proteins in the intrinsic pathway. The finding is exciting because few of the mismatch repair gene was suggested to participate directly in the apoptotic pathway.

6.5 Future work

Tones of works remained to be done if we would like to have a clear picture about how hypoxia/reoxygenation may affect the drug response in cells. The resistance mechanisms of drugs other than doxorubicin and cisplatin in the hypoxia/reoxygenation treated carcinoma cells can be examined by RT-PCR, Western blotting and RNAi interference so as to find out the target gene(s).For example, the resistance mechanism of camptothecin in HepG2 cells may be due to the reduced expression of topoisomerase I while the resistance mechanism of IFNa may be due to the defect in Jak-Stat proteins. For the resistance mechanism of doxorubicin in A10HR and A20HR cells, the role of histone deacetylation in the expression of mdr1 could be investigated by the use of histone deacetylase inhibitor, trichostatin A. For the resistance mechanism of cisplatin in G10HR and G20HR, methylation specific PCR and sequencing of the PMS2 promoter could be carried out in order to evaluate the methylation status of the PMS2 promoter and further confirmed the role of promoter methylation in the expression of mdr1 and P-glycoprotein. Last but not the least, the interaction between PMS2 and Fas could be determined by Luciferase Reporter Assay so that the role of PMS2 in triggering extrinsic apoptotic pathway could be proven. Hopefully, the present study can give some insight into the design of more appropriate treatment strategy for the cancer patients, especially for those bearing solid tumors.

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